THE FRACTIONATION OF VITAMIN B_2 IN MARMITE

by

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INTRODUCTION

The aim of this investigation at its incention was to elucidate the chemical nature of the anti-anaemic substance contained in Marmite. In view of the suggested relationship of vitamin B_2 to this substance it was decided to proceed directly to the isolation of vitamin B_2 from Marmite following the procedure of Kuhn (1933) and to experiment with the pure product in a study of anaemias.

The methods used by Kuhn had at this time been described only as applied to egg white and to whey. Early in our work it became apparent that there were difficulties attending the extraction and isolation of vitamin B_2 from Marmite which had not arisen in the fractionation of whey or egg white with the result that a crystalline flavin pigment was not obtained by us from Marmite. It was now evident that each stage of the fractionation of Marmite would have to be followed by a biological assay of the vitamin B_2 content of the fractions. The researches to be described herein are confined to such a study and it remains for future work to effect their completion before a study of the anti-anaemic activity of the pure product can be carried out.

REVIEW

I. THE RELATION OF VITAMIN B_2 TO ANAEMIAS

The results of many recent investigations of the aetiology and pathology of pernicious anaemia are consistent with the theory that pernicious anaemia is a deficiency disease characterized by the absence from the gastric mucosa of a specified enzyme. By the interaction of this thermolabile principle in gastric juice (variously named "intrinsic factor" Castle (1929), "Addisin" Morris et al. (1932) and "hematopoietin" Wilkinson and Klein (1932)) and beef muscle protein as substrate a haemopoietically active thermostable substance is formed. This product is believed by Klein and Wilkinson (1932) to be identical with the specific anti-anaemic substance contained in liver.

The chemical nature of the "liver active principle" or of its precursor contained in beef protein has not yet been determined. The extrinsic factor was believed by Strauss and Castle (1932) to be vitamin B_2 since the "characteristics and distribution" of the extrinsic factor and vitamin B_2 were strikingly similar and since an autolyzed yeast product (Marmite) after incubation with gastric juice was found by them to be anti-anaemically active.

The original objective of the writer was to examine this suggested relationship between vitamin B_2 and the principle in liver active in pernicious anaemia. Marmite was used successfully by Wills and Naish (1933) in the treatment of tropical sprue, a macrocytic anaemia closely resembling pernicious anaemia but in which the intrinsic factor is believed to be present in the gastric juice. It is now generally accepted that the haema-

topoietic action of autolyzed yeast is in large measure due to its high content of extrinsic factor. At the same time Marmite is the richest known source of the water soluble vitamin B complex. The consensus of evidence (Davidson (1932); Goodall(1932) Ungley (1933); and Wilkinson and Klein (1932)) is however, against a common identity for the extrinsic factor and the growth promoting constituent of the vitamin B complex. Following the recording of several failures to prepare an anti-anaemically active fraction by the interaction of highly purified vitamin B_2 preparations and normal gastric juice (Ungley and James (1934); Diehl and Kuhnau (1933)) Castle (1934) has, in effect, withdrawn his original suggestion in this regard.

While purified vitamin B_2 may not act as the extrinsic factor the evidence, nevertheless, points to some connection as yet undetermined between vitamin B_2 and haematopoiesis. According to Guha (1931) vitamin B_2 deficient rats suffer from an anaemia which does not respond to iron or copper but is cured by liver or yeast fractions. The extensive researches of Goldberger et al (1928) on human pellagra have revealed that this disease results from a lack of, and is specifically cured by a substance contained in yeast, named by him the "P.P.Factor" and now believed to be identical with vitamin B_2. In pellagra there occur degenerative changes of the spinal nerves similar to those found in pernicious anaemia. Rhoads and Miller (1933) have demonstrated a striking resemblance between the syndrome of chronic black tongue with anaemia in dogs usually regarded as analagous to pellagra, and the macrocytic anaemia and clinical features of sprue. The condition was alleviated by substances rich in

vitamin B_2 but not by parenteral administration of large doses of liver extract. It was suggested therefrom that "the dog utilizes vitamin B_2 as such in haematopoiesis and not as an intermediate product of gastric digestion". The glossitis of sprue and of Addisonian pernicious anaemia in man may very well be caused by the lack of some part of the vitamin B complex.

II. THE PHYSIOLOGICAL ACTION OF VITAMIN B_2

The dual nature of vitamin B was first recognized by Goldberger (1926) and later confirmed by other workers. The first differentiation between vitamins B_1 and B_2 as they are now known was effected by autoclaving. Vitamin B_1 is destroyed by autoclaving for one hour at 120°C. with the solution adjusted to pH 8-9, but vitamin B_2 retains its activity. This separation of the two components of the water soluble B complex led to a concerted attack on the problem of further concentrating and purifying vitamin B_2. Chick, Coping and Roscoe (1930) have shown that concentrates of vitamin B_2, free from B_1 may be conveniently obtained from egg-white by removing the heat-coagulable proteins. Guha (1931) obtained a concentrate of vitamin B_2 from yeast by successive fractionations with lead acetate and alcohol. Levene (1932) described a procedure by means of which concentrates of both vitamins B_1 and B_2 may be obtained from brewers' yeast, the latter being found in the residue remaining after applying his silica -extraction process.

Each step in the separation of the two components of the water soluble vitamin B complex was controlled by biological assays which are based on their different physiological actions.

Vitamin B_1 is the antineuritic growth promoting factor and vitamin B_2 the anti-pellagra growth promoting factor. A deficiency of vitamin B_2 in the diet is the cause of pellagra in human beings, of retarded growth and a characteristic dermatitis in rats and chicks and of black tongue in dogs. These symptoms are accompanied by nerve lesions and disturbances of the gastro-intestinal tract.

The effects of vitamin B_2 deficiency on rats and chicks have been studied extensively by numerous workers. In vitamin B_2 studies with rats synthetic diets are used which have been freed from the vitamin B complex and supplemented by vitamin B_1(B_4). The assay method was curative and the growth rates taken as the criterion of activity. With chicks synthetic diets have not been found satisfactory. At present a basal diet of natural foodstuffs is being used (Kline et al. 1932-33) By subjecting this diet to dry heat they claim that vitamin B_2 is destroyed but without any appreciable destruction of the other essential factors. This diet produces a pellagrous like dermatitis as well as retardation of growth. The vitamin B_2 content of a supplement to this diet is measured by its activity to prevent pellagra and to promote growth.

III. THE CHEMICAL PROPERTIES OF VITAMIN B_2

Coincident with these investigations on the physiological action of vitamin B_2 progress was also made toward the elucidation of its chemical nature. Narayanan and Drummond (1930) found that vitamin B_2 could be almost completely adsorbed on Fullers' earth at pH 0.1 but encountered a great difficulty in

removing the adsorbed active factor. This difficulty was overcome by Kuhn, György and Wagner-Jauregg (1933) who succeeded in eluting the active adsorbate with a mixture of four parts of water, one part of pyridin and one part of methyl alcohol. From this elution they prepared a crystalline flavin pigment and claimed to have established its identity with vitamin B_2. Their alleged vitamin B_2 activity of flavin was based on the growth response obtained with rats when the Bourquin-Sherman (1931) vitamin B_2 free diet (supplemented with B_4*) was used. The pellagra-preventing activity of the flavins could not be demonstrated because with this diet the rats did not develop dermatitis. György (1935) has found that the failure of rats to develop dermatitis was due to the fact that the 80% alcohol extract of wheat used as a source of vitamin B_1 in the Bourquin-Sherman diet contained also sufficient amounts of the pellagra-preventing substance. Using a crystalline preparation of B_1 he found that vitamin B_2 is a complex of at least two factors i.e. (a) flavin and (b) the "supplementary factor" (or perhaps factors) contained in Peters's (Kinnersley at al., 1933) "charcoal eluate" from bakers' yeast. The flavin pigment has growth promoting activity only. The pellagra preventing action is a characteristic of the "supplementary factor" which is also growth promoting, but the body weights remain subnormal unless flavin is added.

*Vitamin B_4 is contained in the mercuric sulphate precipitate of a vitamin preparation of B_1 concentrate prepared from yeast according to Kinnersley et al. (1932)

EXPERIMENTAL

A PRELIMINARY ATTEMPT AT THE DIRECT ISOLATION OF THE FLAVIN PIGMENT FROM MARMITE.

The procedure employed by Kuhn (1933) for preparing ovoflavin consists in shaking finely powdered egg albumin with 80% methyl alcohol for 24 hrs. at 30°C. The supernatant is syphoned off and the shaking with more methyl alcohol repeated for 12 hrs. The combined pigment extracts are distilled, acidified with concentrated hydrochloric acid to a pH 0.1 and then are shaken for one hour with Fullers' earth (100gms. to 2 Kgms. egg albumin). The centrifuged adsorbate is washed free of chloride and eluted by shaking 1½ hrs. with a mixture of one part of pyridine, one part of methyl alcohol and four parts of water. It is then centrifuged and washed with 50% methyl alcohol. The eluate and washings are concentrated in vacuo. The additional precipitate of Fullers' earth is centrifuged and washed again. An equal or twice the volume of methyl alcohol is added to precipitate the colloidally dispersed Fullers' earth and if necessary a few drops of glacial acetic acid are added. The Fullers' earth is removed by centrifuging and the supernatant is concentrated in vacuo. Sufficient acetone is now added to precipitate the colourless compounds. The acetone is removed by distillation and the residual solution is extracted with aether until the ether shows no yellow colouration. The yellowish brown aquaeous solution which has a green fluorescence is now quite clear. It is again precipitated by adding acetone until complete precipitation is obtained. Next

day it is filtered and concentrated in vacuo to 20 cc. and again shaken with Fullers' earth for three hours. The adsorption filtrate is still yellow in colour. The Fullers' earth is washed with a little water and shaken for 2½ hrs with a mixture of forty parts of water, twenty parts of methyl alcohol and fifteen parts of pyridin. After centrifuging, the supernatant is concentrated in vacuo to 40 cc. precipitated with acetone and left overnight. The next day the solution is filtered and concentrated in vacuo to 10 cc. A 5% silver nitrate solution is then added until complete precipitation is obtained. The reddish brown precipitate separated by centrifuging is extracted with small portions of 50% acetic acid until the acid layer is colourless. The combined solutions are freed from silver with hydrogen sulphide and centrifuged. The supernatant is concentrated almost to dryness in-vacuo and the crystals which separate out are recrystallized three times in a small amount of boiling 2N. acetic acid. Two kilograms of dry egg albumin (commercial) yield 2 mg. crystalline ovoflavin. The needle shaped crystals are orange-brown in colour, and form round clusters. The empirical formula of flavin is $C_{17}H_{20}N_4O_6$

In our attempts at isolating crystalline flavin from Marmite Kuhn's method as described above was in general adhered to. In the first attempt we used 100gms. and in the second 500 gms. of Marmite. To prepare an 80% alcohol extract the Marmite was dissolved in a minimum amount of water and 95% methyl alcohol added to the desired concentration. For adsorption we used kaolin.

Our failure to obtain a crystalline preparation of flavin from Marmite was probably due to the small amounts of Marmite used. Kuhn obtained only 2 mg. of flavin from 2 Kg. of dry

egg albumin. Since Marmite is a highly concentrated source of vitamin B_2 we assumed that it likewise contained a relatively large amount of flavin. Such may not be the case and in fact Marmite, weight for weight, may contain a much smaller amount of flavin than egg-white or whey powder. Later experiments indicate that in the preparation of the alcohol extract a considerable loss of flavin may have taken place. The main reason for our failure we believe to have been due to the use of kaolin for adsorption. György, Kuhn and Wagner-Jauregg (1934) have found that kaolin, aluminum oxide, kieselguhr or calcium carbonate are unsuitable for the adsorption of flavin. This article appeared after we had discontinued these experiments and had commenced the studies now to be described and in which Fullers' earth has always been used.

II. THE FRACTIONATION OF VITAMIN B_2 IN MARMITE USING CHICKS FOR THE BIOLOGICAL ASSAY.

Throughout the experiments to be described the following conditions were observed. Day-old chicks in groups of 6 to 25 were used. They averaged in weight from 30 gms. to 35 gms. At the end of the first week they were banded with numbered wing bands, weighed, and the weight recorded. Weekly records of weights were kept. The chicks were housed in a well ventilated laboratory kept at 70°-80°F. Large wire cages with raised wire screens were used to prevent coprophagy. The cages were heated with electric light globes painted red to give maximum heat with minimum light.

The basal ration used was that of Kline, Keenan, Elvehjem and Hart (1932) and was composed of:-

Yellow Corn	58 parts	Salt (common)	1 parts
Middlings	25 "	Cod Liver Oil	2 "
Casein	12 "	$CaCO_3$	2 "

The diets were made up in lots of 250 gms. and 500 gms. depending on the number and age of the chicks.

The basal ration was rendered deficient in vitamin B_2 by heating in a dry state for six days. Varying temperatures within the range of 95°C. to 130°C. were used. The ration was spread on trays in layers a quarter of an inch thick and heated in a thermostat oven. Temperature variations were checked from time to time. Only the yellow corn, middlings, casein and salt were treated thermally. The cod liver oil and $CaCO_3$ were added as the diets were prepared for feeding.

Photograph of Chick (Group XXV)

with Fully Developed Symptoms of Pellagra

Marmite, or an extract prepared from Marmite was used as the vitamin B_2 supplement. Marmite is a commercial autolysate of yeast preserved with salt and is of a semi-solid consistency. To insure its even distribution in the diet the following procedure was employed. A small amount of hot water was added to the weighed portion of Marmite and stirred to a thick paste with a glass rod. This paste was slowly diluted until all the Marmite dissolved. The heated basal ration was then added in an amount sufficient to give a thick mush. This mush was then incorporated in the remainder of the diet by rubbing thoroughly to ensure its freedom from lumps.

No feeding experiment was conducted longer than six weeks. The birds were observed daily and examined if necessary. The first signs of pellagra or any other pathological symptoms were recorded. The external manifestations of pellagra are minute lesions at the beak which gradually increase in size as the disease progresses and finally appear as round crusty symmetrical scabs at both corners of the mouth. The eyelids become granular, contract and finally close so that vision is completely impaired. The wing feathers are shed and in general feathering is retarded. The birds are very nervous, somnolent and congregate as close to the lamp as possible. The food consumption is reduced but apparently not as the result of loss of appetite. These symptoms are associated with a characteristic retardation of growth and great mortality.

2nd. Photograph of Chick (Group XXV)
Showing the Cure of Pellagra by Feeding
for twelve days an 80% Alcohol Extract
of Marmite in Amount Equivalent to 8% Marmite

FIRST SERIES

A STUDY OF THE METHOD OF RENDERING THE BASAL RATION DEFICIENT IN VITAMIN B_2 BY HEAT TREATMENT.

According to Kline, Keenan, Elvehjem and Hart (1932) chicks reared on the basal diet of natural foodstuffs heated dry at 100° for six days invariably developed definite symptoms of pellagra at about three weeks of age. No other deficiency resulted from this treatment. They, therefore concluded that a ration so treated was especially valuable, because it eliminates the necessity of preparing a synthetic diet and adding a vitamin B_1 concentrate, which is the usual procedure in vitamin B_2 assays.

Our preliminary experiments with the basal ration heated dry at 95°-100°C. and used immediately, and the same ration used six months after the heat treatment gave widely divergent results. In the first case sufficient destruction of vitamin B_2 was not obtained to warrant our using this ration for assay purposes. In the second case the destruction of vitamin B_2 as indicated by the incidence of pellagra was sufficient to permit the utilization of this ration as a basis for assay. However the characteristic symptoms of pellagra were further complicated by other symptoms due evidently to the destruction of other dietary factors essential to the normal nutrition of the chick. Further studies were necessary, to establish the conditions of heat treatment which would render the basal ration deficient in vitamin B_2 to a degree suitable for our purposes, without impairing the nutritional value of the ration

in any other respect. The following experiment was therefore carried out. Included in these experiments is an assay of the basal unheated ration using chicks of three different breeds. This was necessary since a future supply of chicks all of the same breed was not assured.

OUTLINE OF EXPERIMENT

Group I - Unheated Control Diet
- Group I A White Leghorn Chicks
- Group I B White Plymouth Rock Chicks
- Group I C Rhode Island Red Chicks

Group II - Control Diet Heated at 95°-100°C.

Group III - Control Diet heated at 95°-100°C. and stored.

Group IV - Control Diet heated at 105°-120°C.

Group V - Control Diet heated at 96°-110°C.

RESULTS

The experimental results are assembled in Table I and the growth rates graphed in Fig. I.

NOTES

Group I Unheated Control Diet

A. White Leghorn chicks. The growth of these chicks was a good average for this breed. All the chicks were healthy with one exception, which, in the 5th week, developed a bone condition diagnosed as perosis.

B. White Plymouth Rock Chicks. The chicks were healthy throughout the experiment and show good average growth for this particular breed.

C. Rhode Island Red Chicks. The chicks showed good growth, but four developed perosis.

Group II Control Diet Heated at 95°-100°C.

White Leghorn Chicks. Eight chicks (53%) showed symptoms of pellagra in the fourth week. In the 5th week ten chicks had lesions at the corners of the beaks, retarded feathering, granular eyelids and in two cases the eyes were closed entirely. Growth was subnormal but there was no mortality.

Photograph of Chick (Group V)
with Typical Opisthotonos
Characteristic of Vitamin B_1 Deficiency.

Group III Control diet heated at 95 -100°. used after six months storage

White Plymouth Rock Chicks. From the first week the chicks failed to grow normally. At the end of the second week four chicks (40%) had severe pellagra. A fifth chick developed pellagra at the end of the 3rd week. There was 100% mortality. The gain in weight in four weeks was only 50 grams as compared with a gain of 116 gms. in the same period by chicks of the same breed on the same ration unheated.

Group IV Control Diet Heated at 105°-120°C.

White Plymouth Rock Chicks. Growth was almost completely retarded. Only 25 gms. were gained in four weeks. A 50% incidence of pellagra at the end of the 2nd week and 50% mortality in the first week resulted. By the end of the 4th week all the chicks were dead.

Group V Control Diet Heated at 95° -110°C.

Rhode Island Red Chicks. The average gain in weight during the first three weeks was 25 grams but only 24 grams at the end of the 4th week. There was 85% pellagra at end of the 3rd week which involves all the surviving chicks. Two chicks with slight lesions at the corners of their beaks recovered spontaneously at the end of the 4th week. Four out of seven chicks were dead at the end of the 4th week. One had polyneuritis, another polyneuritis and pellagra, a third pellagra and a fourth was squatting and falling over when attempting to move.

SUMMARY OF RESULTS

A basal ration of natural food-stuffs promotes average growth in chicks of various breeds. When this ration is heated at 100°C. or above some factor is destroyed, in the absence of which chicks developed a pellagrous-like syndrome characteristic of vitamin B_2 deficiency. A basal ration of natural foodstuffs can therefore be rendered deficient in vitamin B_2 by heat treatment.

The chief symptoms of this pellagrous-like syndrome are retarded growth, dermatitis and increased mortality. It was clearly demonstrated that the severity of these symptoms is more marked when the temperature to which the basal ration has been heated is increased. It is possible that the length of

time which the ration is stored after heating may also be a factor in increasing its deficiency. The relative gain in weight of the chicks on the different diets was as follows:

	Group	Gain in weight (gms.) in three weeks
I A	Control unheated diet (White Leghorns)	89
B	Control unheated diet (White Plymouth Rocks)	116
C	Control unheated diet (Rhode Island Reds)	135
II	Control diet heated at 95°-100°C. W. L.	69
III	Control diet heated at 95°-100°C. used after 6 months W. P. R.	56
IV	Control diet heated at 105°-120°C. W. P. R.	23
V	Control diet heated at 96°-110°C. R. I. R.	24

It will be seen that the control diet heated at 95°-100°C. gave slightly retarded growth. The gain in weight of the chicks on this diet for a period of three weeks was 21 gms. less than the gain in weight of the normal chicks, over the same period. Pellagra developed at four weeks and its incidence was 64%. The mortality (13%) indicates that the symptoms are not fatal. This represents a mild condition of vitamin B_2 deficiency.

The control diet heated at 95°-100°C. and stored for six months gave retarded growth from the beginning. The gain in weight of the chicks on this diet for a period of three weeks was 60 grams less than the gain in weight of the normal chicks over the same period. The onset of dermatitis is at two weeks; its incidence is 57% and the mortality 100%. This represents an acute condition of vitamin B_2 deficiency. Storage of the diet after heating increased the destruction of vitamin B_2.

With the control diet heated dry at 105°-120°C. very poor growth resulted. The average gain in weight over the three week period was only 22 gms. The onset of dermatitis is 50% at two weeks and the incidence also 50%. The 50% mortality in the first week and 100% mortality at four weeks indicates that the entire vitamin B complex is lacking in this diet. This heat treatment has destroyed not only vitamin B_2 but in addition some other essential factors.

The control diet heated at 96°-110°C. gave better growth during the first three weeks than the control diet heated at 95°-100°C. and stored for six months. Growth ceased in the 4th week. Judging by the incidence of polyneuritis and other symptoms (which for convenience are referred to as "paralysis") other factors besides vitamin B_2 were affected by the heating. In fact as there was at the same time a spontaneous remission of symptoms of pellagra in two cases it is quite possible that the sudden cessation of growth was due to a deficiency of vitamin B_1 or some factor other than vitamin B_2 and that vitamin B_2 is only partially destroyed by heating at the above temperature. The low mortality from pellagra and the fact that three chicks survived after four weeks points in this direction.

SECOND SERIES

THE VITAMIN B_2 CONTENT OF MARMITE AS DETERMINED BY SUPPLEMENTING THE CONTROL PELLAGRA PRODUCING DIETS WITH VARYING AMOUNTS OF MARMITE.

OUTLINE OF EXPERIMENT

A. Control Diet heated at 95°-100° C.
   Group VI + 2% Marmite
   Group VII + 4% Marmite

B. Control Diet heated at 95°-100° C. and stored six months.
   Group VIII + 6% Marmite

C. 1st and 2nd week - Control Diet heated at 95°-100° C. and stored for six months.
   3rd and 4th week - Changed to Control Diet heated at 130° C.
   5th and 6th week - Changed to Control Diet heated at 105°-120° C.
   Group IX + 4% Marmite throughout
   Group X + 6% Marmite throughout

D. Control Diet heated at 105°-120° C.
   Group XI 1st and 2nd week + 3% Marmite
   3rd week + 4% Marmite
   4th week + 6% Marmite
   Group XII 6% Marmite added throughout
   Group XIII First two weeks + 6% Marmite
   After 2nd Week + 8% Marmite

E. Control Diet heated at 96°-100° C.
   Group XIV + 6% Marmite

RESULTS

The experimental results are assembled in Table II and the growth rates graphed in Figs. II, III, IV and V.

NOTES

Experiment A

Group VI. Control Diet heated at 95°-100° C. + 2% Marmite. White Leghorn chicks receiving this diet showed better growth than chicks of the same breed fed the unheated control diet. There were no pathological symptoms. One chick died in the 2nd week.

Group VII Control Diet heated at 95°-100°C. + 4% Marmite. White Leghorn chicks. The rate of growth greatly exceeded that of the chicks fed the unheated control diet or that of the chicks receiving 2% Marmite (Group VI). There was no mortality and no pathological symptoms.

Experiment B

Group VIII Control Diet heated at 95°-100°C. and stored for six months + 6% Marmite.

White Plymouth Rock Chicks. They showed excellent growth for the first two weeks. The two smallest chicks died in the second week. In the third week four chicks remained squatting and were unable to stand. Their legs were atrophied. In some respects these symptoms resembled those described by Keenan, Kline, Elvehjem and Halpin (1933) and described by them under the general term "paralysis" as follows: "The onset of the paralysis is marked by the preference of the bird to squat rather than stand. If disturbed it will rise, walk a short distance and squat again. In almost all cases a marked atrophy of the leg muscles can be detected. Generally death occurs a few days after the development of these symptoms but chicks that grow slowly may survive two or three weeks." These symptoms occurred in chicks fed a synthetic diet and of average weight of 70 gms. at three weeks of age. Our chicks were fed a diet of natural foodstuffs heated dry at 95°-100°C., stored six months, and supplemented with 6% Marmite, and averaged in weight 84 grams when two weeks old. The four chicks with "paralysis" died shortly after the symptoms developed. The post-mortem showed empty intestines, enlarged and clogged coeca and pale hypertrophied kidneys.

According to Keenan, Kline, Elvehjem and Halpin (1933) birds with "paralysis" show a degeneration of the brain which is "confined in most cases to the cerebrum and in a few cases to the cerebellum. The affected area appears white and caseous and the cells oedematus in character." The brain of one of our chicks which died of "paralysis" was examined by the Provincial Laboratory. They reported that sections of the brain and cerebellum showed no obvious abnormalities.

Keenan et al. ascribe this paralysis to a deficiency of vitamin B_4 or a substance closely related to it chemically and physically. They observed several cases of paralysis in groups of chicks reared on grain rations. The majority of these cases were encountered with grain that had been stored for several months. The addition of 8-12% yeast to unpurified synthetic diets did not correct the deficiency but 18% vacuum-dessicated hog liver did.

Three more chicks died in the 3rd week. Only one chick survived. This chick showed a remarkably rapid rate of growth. At the end of six weeks it weighed 407 gms. The average weight at this age for chicks of the same breed on the unheated basal control was 337 gms.

Experiment C.

1st and 2nd week - Control Diet heated at 95°-100°C. and stored six months.
3rd and 4th week - changed to control diet heated at 80°
5th and 6th week - changed to control diet heated at 105°-120°C.

Group IX. + 4% Marmite

White Plymouth Rock chicks. The growth was better than average during the first two weeks. After the second week growth was retarded and was poorest in the 5th week when the average gain in weight was only 6 gms. as compared with the normal rate of 80 gms. for that period and for that breed of chicks. After the 5th week the rate of growth increased very rapidly. The mortality was 57%. One chick died in the 1st. week and a second in the 4th week. In the 5th week a death from haemorrhage occurred and another in the 6th week. Three chicks survived till the end of the experiment. Their average weight was 181 gms. at six weeks.

Group X. Same diet as group IX + 6% Marmite.

White Plymouth Rock chicks. Growth was excellent during the first two weeks. The rate of growth declined from the 2nd to the 5th week, becoming again more rapid in the 6th week. The mortality was 50% but there were no deaths during the first three weeks. One chick was observed to squat, turn round and round and to fall over on its side. It preferred to sit propped up against any support. A similar condition has been observed in rats by Haliday (1934) and was attributed by her to a deficiency of a factor found in whole wheat, which closely resembles B_4 but is not identical with it.

A second chick was also observed squatting. In this case the condition more closely resembled the chick "paralysis" described by Keenan. Examination also revealed a subcutaneous haemorrhage of the left breast and under the left wing. At death there was a haemorrhage from the mouth. These two chicks died when three weeks old. Two more cases of haemorrhage occurred. One in the 5th week which ended fatally and another in the 6th week. Similar haemorrhages were observed and described by Dam and Schönheyder (1934) in a study of a deficiency disease in chicks resembling scurvy. This disease occurs in chicks reared on a certain artificial diet containing 10% Marmite but was not observed when the starch in this diet was replaced by a mixture of cereals. According to these authors ascorbic acid given by mouth or subcutaneously does not prevent the disease. The disease is ascribed to the lack of a factor or factors occurring in cereals. McFarlane, Graham and Richardson (1931) first described this haemorrhagic condition in chicks and observed the failure of the blood to clot. These haemorrhages did not occur when the protein of the diet was caseinogen which had been extracted with ether. When ether-extracted white fish meal or ether extracted meat powder was used in lieu of caseinogen the haemorrhages occurred. Rations containing fish meal and meat powder which had not been extracted with ether did not cause haemorrhages.

In our experiments these haemorrhages occurred in chicks receiving a mixture of cereals and casein which had been subjected to heat treatment at various temperatures.

Experiment D. Control diet heated at 105°-120° C.
Group XI. 1st and 2nd week + 3% Marmite
3rd week + 4% Marmite
4th week + 6% Marmite

White Plymouth Rock Chicks. Growth was retarded from the beginning. The rate of growth in the first two weeks was similar to that of Group III, i.e. fed the control diet heated at 95°-100° C. and used after six months. It increased slightly when the percentage of Marmite was raised in the 3rd and 4th week. There was 65% mortality in the first week and 100% at the end of five weeks. The mortality in the first week was greater than that of chicks fed the unsupplemented control diet heated at 105°-120° C. Two chicks developed pellagra and died in the 3rd week. One chick died following a haemorrhage in the 5th week.

Group XII* Control Diet heated at 105°-120° C. + 6% Marmite.
Rhode Island Red Chicks. The growth of these chicks was retarded. Their average weight at five weeks was the same as that of the chicks of this breed fed the unheated basal ration at four weeks. The same relation exists as that already found between the growth rates of White Leghorn chicks receiving the control diet heated at 95°-100° C. and the same breed of chicks fed the basal unheated diet. (Group I A and Group II). In that experiment 2% Marmite gave better than normal growth. This indicates that with Group XII 7% or 8% Marmite would have been sufficient to insure normal growth and that the control diet heated at 105°-120° C. contains one seventh or one eighth of the amount of vitamin B_2 contained in the control ration heated at 95°-100° C. This deduction is not significantly affected by the fact that different breeds of chicks were involved. The mortality was 71%. Three chicks died when two weeks old and two died in the 4th week. One of these showed severe pellagrous dermatitis and haemorrhages of the right leg and right side. Another case of haemorrhage combined with an abdominal oedema occurred in the fifth week. The chick

*The haemorrhages described above occurred when 4% or 6% Marmite was fed. In addition to the salt in the basal ration Marmite contains 7.5% of salt. It was thought possible that the oedema and haemorrhages resulted from too high a salt content of the diet. The salt content of this and of all subsequent diets was adjusted to 1% allowing for the salt in the Marmite supplement.

Photograph of Chick (Group XIV)
Suffering from Paralysis.

survived till the end of the experiment. Dam and Schonheyder have also observed that the haemorrhages were often absorbed in the course of a few days and the birds could be reared up to the age of two months (the end of the experiment.)

Group XIII. Control Diet heated at 105°-120°C.
First two weeks + 6% Marmite
After two weeks + 8% Marmite

Rhode Island Red chicks. For two weeks the chicks of this group were reared together with the chicks of group XII. The early and rather large incidence of pellagrous dermatitis (35%) at the end of the second week was evidence that 6% Marmite was not sufficient to supply the vitamin B_2 deficiency of the heated control diet. The birds were, therefore, divided into two groups. Six chicks (one died early) were left as control on 6% Marmite and eleven were given 8% Marmite. The eleven chicks placed on 8% Marmite included all which had already dermatitis.

Supplementing with 8% Marmite gave improved growth at the end of the 3rd week or one week after the change of diet. Their average weight at that time was 94 gms. as compared with an average of 87 gms. for the chicks still receiving 6% Marmite. They continued gaining rapidly and at the end of the 5th week their average weight was 201 gms. i.e. 50 gms. less than that of the chicks of the same breed fed the basal unheated diet. The difference is explainable as being due to the large number of sick chicks. It has been our experience throughout these experiments that once a chick develops dermatitis it never resumes quite normal growth, after being changed to a diet giving complete protection.

The mortality of this group was 18% as compared to 71% mortality in Group XII. Of the chicks with pellagrous lesions which were transferred from Group XII two died at the end of the 3rd week, one recovered at the end of the third week and three recovered at the end of the 4th week. There were two cases of slight haemorrhage at the end of the 5th. week.

Experiment E.

Group XIV Control Diet heated at 96°-110°C. + 6% Marmite.

The average weight of this group of chicks at three weeks was only slightly lower than that of the chicks receiving the corresponding unheated basal ration and slightly higher than the weights of the chicks fed the control diet heated at 105°-120°C. supplemented with 6% Marmite. At the end of the 4th week there was a sudden slowing of the growth rate.

There was no pellagra but there were two cases of "paralysis". The syndrome of paralysis is characterized by squatting, falling to one side, or falling with head forward and bent downward as if unable to hold up the head, toes extended and the head bent down between them. The bird keeps moving the head from side to side and emits sounds of distress as if in pain. At the onset of the condition the birds fly rather

than walk but later are too weak to do either. One of these cases was fatal.

COMMENTS ON RESULTS

When added in sufficient amounts Marmite will adequately supplement a basal ration of natural foodstuffs rendered deficient in vitamin B_2 by heating dry at 100 C. or above. Marmite therefore, contains vitamin B_2.

When a ration, rendered deficient in vitamin B_2 by heating at 95°-100°C., is fed to chicks their average weight at five weeks is the same as the average weight at four weeks of the same breed of chicks on the unheated basal ration. Two per-cent Marmite is more than sufficient to make good this deficiency. It has been found, as will be seen later, that this relation of weights is an approximate expression of the growth response to 2% Marmite.

The appearance of pellagrous dermatitis either follows a slow continuous retardation of growth or a sudden rapid loss of weight. A sufficient supply of the growth factor gives also protection from pellagrous lesions. The quantitative relationship suggested above would appear to apply both to growth and to the dermatitis.

Four per-cent Marmite as supplement to a ration heated at 100°C. gives an average weight at four weeks equal to that of chicks at five weeks receiving the basal unheated control. This is an indication that in this case we have a surplus of vitamin B_2 equivalent to that contained in 2% Marmite. The results with Group VI support this conclusion. The results obtained with Group VII also indicate that the chick has an

optimum requirement of vitamin B_2.

Six per-cent Marmite added to a control diet which has been heated at 95°-100° C. and stored six months after heating gives better than average growth and complete protection from dermatitis. Four per-cent of Marmite would most likely have been sufficient. As 2% Marmite was more than sufficient to supplement the deficiency of vitamin B_2 in this same diet six months before, this is additional proof that storing increases the destruction of this factor. Storage of the heated diet appears to have resulted in the destruction of factors other than vitamin B_2 since chicks on this diet developed "paralysis". The "paralysis" and haemorrhage will be discussed after all the cases have been presented.

In Groups VIII and IX we employed three diets of three different degrees of deficiency and supplemented with 4% or 6% Marmite. We observed that in the 3rd week although the diet being used at this time was more deficient in vitamin B_2 than the preceding one the growth is still good and did not decline until the 4th week. The rate of growth was less with 4% Marmite than with 6% Marmite and least in the 5th Week. This would indicate a storage of vitamin B_2 in the body.

Three, four and six per-cent Marmite added to a ration heated at 105°-120°C. does not prevent pellagra. When 8% Marmite is used however complete protection results. The deficiency of the ration heated at 95°-100°C. was fully supplemented by 2% Marmite (Group II), the same ration stored for six months required 4% Marmite (Group III) and now a ration heated at 105°-120°C. is seen to require supplementing by 8%

Marmite. Storing the heated ration destroyed about one-half of the vitamin B_2 in the basal ration. Heating to 120°C. seems to destroy completely the vitamin B_2 in the basal ration. In this latter case the high mortality indicated a heat destruction of the entire vitamin B complex. As an approximation we may say that 8% Marmite contains the same amount of vitamin B_2 as the basal unheated ration or that the vitamin B_2 content of Marmite is about 12 times as great as that of the unheated basal ration.

When 6% Marmite was added to the ration heated at 105°-120°C. the average weights of the chicks at five weeks was the same as that at four weeks of the chicks on the basal unheated ration. When 8% Marmite was added the protection was complete. This further supports our contention that there is a definite quantitative relationship between the rate of growth of chicks and the vitamin B_2 content of the diet.

We have made some preliminary observations on the effect of heating and of autoclaving on the vitamin B_2 content of Marmite. Elvehjem, Kline, Keenan and Hart (1932-33) found that heating yeast at 95°-100°C. for six days destroyed half its content of vitamin B_2. Block and Farquhar (1933) claim that heating yeast at 95°-100°C. for two or four weeks does not impair its vitamin B_2 activity.

We heated Marmite at 120°C. for six days. The slight destruction of vitamin B_2 in the basal ration when heated at 100°C. was the reason for using a temperature of 120°C. in the heating of Marmite. The Marmite was first dried at 100°C. for four days, reduced to a powder and then heated at 120°C.

for six days. Marmite thus treated was added to the control diet heated at 95°-100°C. (stored for six months) in amounts equivalent to 6% unheated Marmite. The autoclaving of Marmite was carried out at 120°C. and 15 lbs. pressure for five hours. The autoclaved Marmite was added to the same control diet in amounts equivalent to 6% untreated Marmite. White Plymouth Rock chicks were used. The results are summarized in the following table:

	Marmite Heated Dry at 105°-120°C. for six days				Marmite autoclaved at 120°C. for five hours			
Weeks	Weight in Grams	Pellagra	Mortality	Food Consumption	Weight in Grams	Pellagra	Mortality	Food Consumption
1	41		1	30	53			40
2	51	2	1	45	70		1	57
3	63	3	2	55	97	-2	2	65
4	82		2	84			2	
5	123			71				
6	136			71				
		71%	86%	356		40%	100%	162

The average weights of the chicks receiving the heated Marmite are lower than the corresponding weights of chicks on the heated control diet alone. This indicates that the dry heating of Marmite at 120°C. for six days not only destroys its vitamin B_2 content, but also results in the formation of substances having a depressing effect on growth. The mortality was greater than the incidence of pellagra. The water content of Marmite was found to be approximately 26%.

The average weights of the chicks receiving autoclaved Marmite are better than the corresponding weights of the chicks fed the heated control diet alone, but lower than the weights

of the chicks fed the autoclaved basal ration for the same period of time. Their weight at three weeks is the same as the weight at four weeks of the chicks receiving the heated control diet. This indicates that an amount of autoclaved Marmite equivalent to 0% Marmite affected growth to the same extent as 2% Marmite or that autoclaving destroyed two thirds of the vitamin B_2 in Marmite. Chick and Roscoe (1930) Chick Hoping (1930) and M. M. Roscoe (1932) found that autoclaving Marmite at 120°C., 15 lbs. 5 hrs. at its natural pH destroys half its vitamin B_2 content. The fact that the Marmite used in our experiment had been stored in the laboratory for three years prior to its use may have affected these results.

The mortality was 100%. It is a well established fact that autoclaving a ration at 120°C. under 15 lbs. pressure for 5 hours destroys its total vitamins B_1 and B_4 content. The heated control diet used in this experiment has already been shown (Group III) to be deficient in more than one respect. One chick receiving autoclaved Marmite showed the characteristic opisthotonos of polyneuritis. This shows that the heated control diet used in this experiment was deficient not only in vitamin B_2 but also in vitamin B_1.

[illegible]

[illegible] SOLUBILITY OF VITAMIN B_2 IN 80% AND IN 80% ALCOHOL

A preliminary experiment in which a control pellagra producing diet was supplemented with an 80% methyl alcohol extract of Marmite gave negative results. Kuhn and his co-workers (1933) claim that the flavin pigment, isolated and identified by them as the growth promoting vitamin B_2 is soluble in 80% Alcohol. The always use 80% alcohol in their purification of the flavin pigment. Day(1934) on the other hand found that 80% alcohol extracts only about 20% and 60% alcohol extracts approximately 50% of the vitamin B_2 in yeast. Our own results as well as the widely divergent data in the literature regarding the solubility in alcohol of vitamin B_2 made it desirable to establish this point definitely before proceeding further with the purification of this vitamin.

Two methods were used in the alcohol extraction of Marmite. The first method consisted in dissolving Marmite in the least possible amount of boiling, distilled water and boiling eight minutes with constant stirring. The volume of the solution was measured and an amount of 95% alcohol necessary to give a final concentration of 60% or 80% alcohol was added. A precipitate formed, but complete precipitation was only obtained after allowing the suspension to stand overnight, because if centrifuged immediately more precipitate formed and the centrifuging had to be repeated. The precipitate was gummy and could not be completely separated either by centrifuging or filtering. It was dark brown in colour and great difficulty was experienced

in effecting the complete extraction of pigment from it by washing. As we were proceeding on the assumption that the vitamin B_2 activity was contained in the pigmented portion of the Marmite, the extraction of all the pigment was of the utmost importance.

Owing to the large volume of washings there was a great volume of liquid to be freed from alcohol by distillation thus making the process tedious and lengthy. Filtering in place of centrifuging did not simplify the procedure and on the whole we preferred centrifuging. The first two extracts one an 80% alcohol extract, and the other a 60% alcohol extract of Marmite were prepared according to the above method. The 80% alcohol extract was inactive when amounts equivalent to 4% Marmite were added to the control diet heated at 95°-100°C. and tested as a curative agent on pellagrous chicks. The 60% alcohol extract gave better than average growth and a complete protection from pellagra when amounts equivalent to 6% Marmite were added to the control diet heated at 95°-100°C. and stored for six months.

While freeing the above extracts from alcohol by distilling in-vacuo it was noticed that a white precipitate formed in the distilling flask as the alcohol concentration decreased. This indicated the presence in the solution of a substance soluble in alcohol but less soluble in water. We proceeded therefore to experiment by extracting Marmite separately with water and with alcohol.

It was found that when Marmite was dissolved in an equal weight of boiling water and the solution kept on a boiling

water-bath for 8-10 minutes, stirring constantly, a precipitate formed. After standing in the refrigerator overnight this precipitate centrifuged sharply. The precipitate was light brown in colour and a few washing with small amounts of water sufficed to remove practically all the pigment. If the washings were continued long enough to effect complete removal of the pigment the precipitate could not be separated sharply from the supernatant but remained in suspension. When Marmite was dissolved in five times its weight of boiling distilled water, left overnight and centrifuged, the amount of the precipitate was the same, but it was colourless. As only a few washings were needed, however, to remove the pigment where an equal amount of water was used and the final volume of water was smaller, it was decided to adopt the technique of extracting Marmite with an equal weight of water.

The volume of the combined supernatant and washings of the aqueous extract of Marmite was then measured. The aqueous extract was poured with constant stirring into an amount of 99% alcohol to give a final concentration of either 60% or 80% alcohol, and centrifuged. A clear supernatant containing all the pigment was obtained. The separate fractionation with water and alcohol greatly simplified the preparation of the alcohol extracts of Marmite and effected quantitative extraction of the pigment. The 80% alcohol extraction precipitated about one third of the solids of Marmite. This degree of concentration could not be obtained with the 60% alcohol extraction.

The combined supernatant and washings of the alcohol

extraction were freed from alcohol by concentration in vacuo below 60°C. The almost dry residue was taken up in an amount of hot distilled water just sufficient to dissolve it. The residue from an 80% extract of 300 gms. of Marmite could be dissolved in an amount of boiling water to give a final total weight of 300 g. On the other hand to dissolve the residue from a 60% alcohol extract of 300 gms. of Marmite a greater amount of boiling water was required giving a final weight of 500 g.

The extracts so prepared were incorporated in the various vitamin B_2 deficient diets in amounts equivalent to varying percentages of Marmite.

OUTLINE OF FEEDING EXPERIMENT

Group XV

Control Diet heated at 95°-100°C., fed alone until Pellagra developed. An amount of an 80% alcohol extract of Marmite equivalent to 4% Marmite added to the heated control diet during the 6th and 7th week.

Group XVI

Control diet heated at 95°-100°C. and stored six months. Supplemented with an amount of a 60% alcohol extract of Marmite equivalent to 6% Marmite.

Group XVII

Received a 60% alcohol extract of Marmite equivalent to 6% Marmite added to the heated control diets changed as follows:

1st and 2nd week - Control Diet heated at 95°-100°C. and stored six months

3rd and 4th week - Control Diet heated at 130°C.

5th and 6th Week - Control Diet heated at 105°-120°C.

Group XVIII

Same as Group XVII, with the exception that an amount of an 80% alcohol extract equivalent to 6% Marmite was used.

Group XIX

Control Diet heated at 105°-120°C. Supplemented with a 60% alcohol extract of Marmite equivalent to 3% Marmite. After three weeks changed to an amount of the alcohol extract equivalent to 8% Marmite.

Group XX

Same as Group XIX, but supplemented with a 60% alcohol extract of Marmite equivalent to 6% Marmite. After three weeks changed to an amount of the alcohol extract equivalent to 8% Marmite.

Group XXI

Same as Group XX, but fed an 80% alcohol extract equivalent to 8% Marmite.

Group XXII

Commenced on the same diet as Group XXI, but at the end of the 2nd week the diet was supplemented with an 80% alcohol extract of Marmite equivalent to 10% Marmite.

Group XXIII

Control diet heated at 96°-110°C. Supplemented with an 80% alcohol extract of Marmite equivalent to 8% Marmite.

RESULTS

The experimental results are summarized in Table III and graphed in Figs. VI, VII, and VIII.

Group XV Control diet heated at 95°-100°C., fed alone for five weeks until pellagra developed. An amount of an 80% alcohol extract of Marmite equivalent to 4% Marmite added to the heated control diet during the 6th and 7th week.

White Leghorn Chicks. The growth of these chicks was subnormal. Their average weight at five weeks was only slightly higher than the weight at four weeks of the same breed of chicks fed the basal unheated ration. Growth was still subnormal in the 6th week and at the end of the 7th week the average weight was the same as that of chicks receiving the unheated basal ration at five weeks.

Three chicks had pellagra at the end of the 5th week and at the end of the 7th week the fourth chick developed pellagra. There was no mortality.

Group XVI Control diet heated at 95°-100°C. and stored for six months. Supplemented with an amount of a 60% alcohol extract of Marmite equivalent to 6% Marmite.

White Plymouth Rock Chicks. The average weights of this group are better throughout the experiment than the corresponding weights of the chicks of the same breed fed the unheated basal ration. They are the same at the end of six weeks as the weights of the surviving chick on the same heated control diet supplemented with 6% Marmite. The mortality was 40%. One chick died when two days old and another in the 3rd week. There was no pellagra. One chick had a swelling and haemorrhage of the left hock when two weeks old but recovered in the third week. At the end of the 4th week the same chick had greatly swollen cheeks showing subcutaneous haemorrhages. These symptoms subsided during the next two weeks but did not disappear entirely at the end of the experiment. In the 4th week one chick showed symptoms of squatting and turning round and round. Yeast extract (Harris)* was administered and the chick recovered the next day. There was also a fatal case of haemorrhage in the same week and another of squatting and turning. A postmortem on this chick showed no visible pathological changes. Another chick with a swelling and haemorrhage of the right hock recovered and survived till the end of the experiment. There were two cases of perosis in the 5th week and one in the 6th week.

Group XVII Received a 60% alcohol extract of Marmite equivalent to 6% Marmite added to the heated control diet changed as follows:

1st and 2nd week - Control diet heated at 95°-100°C. stored six months
3rd and 5th week - Control diet heated at 130°C.
5th and 6th week - Control diet heated at 105°-120°C.

*Harris Yeast extract is the Osborne and Wakeman (1919) fraction and is a concentrate of vitamin B_1 and B_4.

White Plymouth Rock Chicks. The average weights of this group during the first two weeks were better than the corresponding weights of the chicks (Group 1B) on the unheated basal ration and the same as the weights of the chicks fed 6% Marmite as supplement to the same heated control diets. (Group X) At the end of the third week the average weight of this group was the same as the weight of the chicks on the unheated basal diet and slightly higher than the weight of the chicks on the same heated control diets supplemented with 6% Marmite. At the end of the 4th week the weight on this diet is much lower than on the unheated basal diet, but not appreciably lower than on 6% Marmite. Growth continues subnormal to that with the unheated basal diet till the end of the 6th week (the end of the experiment) but in the 5th week is better than that obtained with 6% Marmite added to the same heated control diets. The average gain at the end of six weeks is 285 gms. on the unheated basal diet, 117 gms. on the three different heated control diets and 6% Marmite, and 113 gms. on the same heated controls used with an amount of a 60% alcohol extract of Marmite equivalent to 6% Marmite.

The mortality was 71%. There was no pellagra but there were four cases of haemorrhage in the 4th week. Two chicks showing haemorrhages died in the 4th week and one in the 5th week.

Group XVIII Same as Group XVII with the exception that an amount of an 80% alcohol extract equivalent to 6% Marmite was used.

White Plymouth Rock Chicks. The growth rate of the chicks in this group was practically the same as Group XVII. The average gain in weight at the end of six weeks was 114 gms. i.e. one gram more than the corresponding gain for Group XVII. The mortality was 73%. Half of the deaths occurred in the first week. The surviving chicks were healthier than in Group XVII. There was no pellagra, but two cases of slight haemorrhages of the wings and neck occurred in the 4th week, neither of them fatal. The symptoms subsided before the end of the experiment. There was also a fatal case of squatting in the 3rd week.

Group XIX Control diet heated at 105°-120°C. Supplemented with a 60% alcohol extract of Marmite equivalent to 3% Marmite, after three weeks changed to an amount of the alcohol extract equivalent to 8% Marmite.

White Plymouth Rock Chicks. The average weight of these chicks in the first week was the same as the corresponding weight of the chicks on the heated control diet alone. In the second, third and fourth week the weight approximated that of the chicks on the heated control diet supplemented with 3%, 4% and 6% Marmite. In the 5th week the weight was the same as that of the chicks on the unheated basal diet at four weeks and at six weeks was the same as the weight at five weeks of the chicks on the unheated basal diet. The mortality was 60%. Two chicks died in the second week. One with slight pellagrous

dermatitis died in the 3rd week. There were three cases of pellagra in the 1st, 2nd and 3rd week. Two of these recovered in the fifth week and gained in weight steadily till the end of the experiment.

Group XX Same as Group XIX, but supplemented with a 60% alcohol extract of Marmite equivalent to 6% Marmite, after three weeks changed to a supplement equivalent to 8% Marmite.

White Plymouth Rock Chicks. The average weight of these chicks in the 1st and 2nd week was the same as the corresponding weight of the chicks on the same heated control diet supplemented with 6% Marmite. The average gain in weight of the chicks in Group XX at four weeks was the same as the corresponding gain in weights of the chicks on the same heated control diet supplemented with 6% Marmite. The gain was only 3 gms. in the 5th week as compared with a gain of 48 gms. by the chicks receiving the 6% Marmite supplement. At the end of the 6th week the weight of these chicks (group XX) was the same as that at four weeks of the chicks on the unheated basal ration. The mortality (71%) was exactly the same as the mortality of the chicks on the same heated control diet supplemented with 6% Marmite (Group XII). There were two cases of pellagra in the 2nd week, both terminating fatally, and two cases of haemorrhage, one fatal and the other recovering.

Group XXI Same as Group XX, but receiving an 80% alcohol extract equivalent to 8% Marmite.

Rhode Island Red Chicks. The average gain in weight of these chicks at the end of the experiment (five weeks) was exactly the same as the corresponding gain in weight of chicks of the same breed on the same heated control diet supplemented with 6% Marmite for the first two weeks and changed to 8% Marmite after the second week. The mortality was 50%. More than half of the deaths occurred in the second week. There were two cases of slight dermatitis in the 3rd week which recovered spontaneously in the 4th week, and four cases of haemorrhage after the 3rd week. The two most severe cases of haemorrhage died.

Group XXII Commenced on the same diet as Group XXI, but at the end of the 2nd week the diet was supplemented with an 80% alcohol extract of Marmite equivalent to 10% Marmite.

Rhode Island Red Chicks. The gain in weight of these chicks at the end of the experiment (five weeks) was the same as the corresponding gain in weight of the chicks in Group XXI or of the chicks on the same heated diet supplemented with 6% and 8% Marmite. There was no mortality and no pathological symptoms.

Group XXIII Control diet heated at 90°-110°C., supplemented with an 80% alcohol extract of Marmite equivalent to 8% Marmite.

Rhode Island Red Chicks. The average weights of these chicks during the first three weeks were practically the same as those of the chicks on the unheated basal diet. (Group I C) At the end of the 4th week however, the rate of growth slowed down and the gain in weight in that week was 29 gms. as compared with 58 gms. gained during the same period by the chicks on the unheated basal control diet. There was no pellagra, no other pathological symptoms and no mortality.

COMMENTS ON RESULTS

A 60% alcohol extract of Marmite added to control diets heated at temperatures ranging from 95°-130°C. had the same effect on growth and incidence of pellagra as did equivalent amounts of Marmite. This indicates that the factor or factors present in Marmite which promote growth and prevent pellagra are completely soluble in 60% alcohol.

An assay of the 60% alcohol precipitate from Marmite gave the following results;

Control Diet heated at 95°-100°C., stored for six months supplemented by the 60% Alcohol precipitate in an amount equivalent to 3% Marmite.

Weeks	Body Weight in grams	Incidence of Pellagra	Mortality	Food Consumption in grams
1	42		2	28
2	58	2	1	59
3	79		2	78
4	102	3	3	
		63%	100%	

The average weights of the White Plymouth Rock chicks receiving the 60% alcohol insoluble residue were practically the same as those receiving the heated control diet alone (Group III) The average gain in weight for four weeks was 60 gms./when fed the residue and 56 gms./when fed the unsupplemented heated control diet. The incidence of pellagra was slightly higher in the group

receiving the residue. In both groups the mortality was 100%. The residue was quite inactive. This proves that 60% alcohol extracts all the growth promoting and pellagra preventing substances present in Marmite.

When control diets heated at three different temperature ranges were supplemented with Marmite or an equivalent amount of a 60% alcohol extract of Marmite the average gain in weights for six weeks with the Marmite was 117 grams and with the 60% alcohol extract 113 grams. There was no pellagra in the chicks fed either supplement. This is further evidence that the vitamin B_2 content of a 60% alcohol extract of Marmite is the same as that of Marmite.

When control diets heated at three different temperature ranges were supplemented with Marmite or an equivalent amount of an 80% alconol extract or an equivalent amount of a 60% alcohol extract the average gains in weight for six weeks on the three supplements were respectively 114 gms., 117gms. and 113 gms. The average weekly weights of chicks on the 60% alcohol extract and the 80% alcohol extract were almost identical. There was no pellagra. This indicates that contrary to our previous findings vitamin B_2 as contained in Marmite is completely soluble in 80% alcohol.

When a control diet heated at 105°-120°C. was supplemented for the first two weeks with 6% Marmite and for the next three weeks with 8% Marmite the average gain in weight at the end of the 5th week was 150 gms. The same gain in weight over the same period of time and on the same heated control diet was obtained with an 80% alcohol extract of Marmite equivalent to 8% Marmite. There was no pellagra on either 8% Marmite or on

an equivalent amount of an 80% alcohol extract of Marmite.

It is evident, therefore, that for purposes of isolating vitamin B_2 from Marmite either 60% or 80% alcohol can be used. There is, however, an important advantage in using 80% alcohol since it precipitates a greater amount of inert material and so gives a greater purification of the active substance.

These observations were confirmed at the time of writing by Elvehjem and Koehn Jr. (1935). Their studies show vitamin B_2 as contained in liver to be soluble in concentrations of alcohol as high as 90% and that high concentrations of alcohol do not have a destructive action on this vitamin.

We have already mentioned (page 12)that the evaluation of our results with Marmite and its various alcohol extracts was complicated by the occurrence of haemorrhages and at least three types of "paralysis". As haemorrhages do not occur in any subsequent experiments our observations in this regard may be summarized now.

We found that haemorrhages did not occur in feeding trials either with the unheated or with the various heated unsupplemented control diets. They did not occur on feeding the supplemented control diets heated at 95°-100°C. or at 96°-110°C. They appeared when a control diet heated at 95°-100°C. and stored for six months or a control diet heated at 105°-120°C. was supplemented with Marmite or with alcohol extracts of Marmite. Their onset was at four weeks or later when Marmite was used and as early as the second week when equivalent amounts of the alcohol extracts of Marmite were fed. With a diet heated at 120°C. and supplemented with 6% Marmite the haemorrhage was more severe and even fatal, but on feeding the same heated

control supplemented with 8% Marmite it was very slight. In the group receiving the same heated control supplemented with an alcohol extract of Marmite equivalent to 8% Marmite there were four cases of haemorrhage, one of these fatal, but on feeding the equivalent of 10% Marmite there were no haemorrhages. Therefore the amount fed of Marmite or alcohol extract of Marmite influences the incidence and the severity of haemorrhages, and further the salt content of the diet is not a contributing factor.

Prolonged storage of a ration heated at 100°C. or heating a ration at 120°C. seems to destroy the factor which prevents the occurrence of haemorrhages in chicks. The amount of this factor destroyed in the basal diet by the above treatment is contained in 10% Marmite or an equivalent amount of an alcohol extract of Marmite. The rate of growth seemingly affects the incidence of haemorrhage. Indicative of this is the absence of haemorrhage on the above mentioned heated controls when used alone and the great incidence of haemorrhage when better growth is obtained by supplementing with an alcohol extract of Marmite equivalent to 8% Marmite. This was the case with the control heated at 95°-100°C. and stored for six months (Group XVI) It seems therefore that the more rapid the growth rate the greater is the requirement for the dietary factor which prevents these haemorrhages. With an adequate supply of vitamin B_2 growth progresses in spite of the appearance of haemorrhages. The haemorrhage preventing factor has no growth promoting properties. In a total of 84 chicks there were 26 cases of haemorrhage, 13 of which were fatal.

FOURTH SERIES

THE ADSORPTION OF VITAMIN B_2 ON FULLERS' EARTH OR ON LLOYD'S REAGENT* FROM ALCOHOLIC EXTRACTS OF MARMITE

The solubility in 60% and in 80% alcohol of vitamin B_2 as contained in Marmite being now established we proceeded to the next step in the isolation of the growth promoting and pellagra preventing factor, namely the adsorption on Fullers' earth and the elution therefrom with dilute methyl alcohol-pyridin mixture. The method followed was that of Gyorgy, Kuhn and Wagner-Yauregg (34) but applied to Marmite in a slightly modified form.

The procedure consisted in first extracting the Marmite with water and precipitating the aqueous extract with 60% or 80% alcohol and freeing it from alcohol by concentration in-vacuo as already described. The aqueous solution of the residue diluted to twice the original weight of Marmite was acidified with concentrated hydrochloric acid (37 cc. of acid to 450 cc. of the extract) to give an N/1 HCl solution.

The proper normality of the solution is the most important condition of adsorption on Fullers' Earth. The acidified aqueous extract was then shaken for one hour with Fullers' earth (25 g. Fullers'earth to an amount of the extract equivalent to 500 gms. of the original Marmite). Next day the suspension was centrifuged and freed from chloride by washing repeatedly until the centrifugate acidified with dilute HNO_3 failed to give a precipitate with $AgNO_3$. The pigment adsorbed on Fullers' earth was eluted by shaking for 1½ hrs. with a

*Lloyd's reagent is an activated Fullers' earth.

mixture of pyridin (1 part) methyl alcohol (1 part), and distilled water (4 parts). The Fullers' earth was removed by centrifuging and washed with 50% methyl alcohol. The eluate combined with the washings was concentrated to approximately 60 cc. by distilling in-vacuo at a bath temperature never above 60°C. The Fullers' earth which separated during distillation was separated by centrifuging. There was still colloidally dispersed Fullers' earth in the supernatant. This was precipitated by adding an equal volume or two volumes of absolute alcohol, again separated by centrifuging and thoroughly washed with 50% alcohol. The combined supernatant and washings were concentrated in-vacuo to 30 cc. and finally diluted with distilled water so that 1 cc. of the solution corresponded to 5 gms. of Marmite.

The activity of the first two elutions prepared as above was negligible. Slightly active eluates were of no value for the further fractionation of vitamin B_2. To determine where the loss of activity occurred an assay of the vitamin B_2 content of the adsorption filtrates was carried out. The combined filtrates and washings from Fullers' earth adsorption were distilled in-vacuo almost to dryness. The residue was dissolved in water so that 1 cc. of the solution contained the equivalent of 1 gm. of Marmite. Feeding experiments showed the adsorption filtrates were active. The probable explanation of the loss of activity during adsorption and elution was that the Fullers' earth used had a poor adsorption capacity. We decided to try adsorption on Lloyd's Reagent. The adsorption on Lloyd's Reagent and the elution therefrom was carried

out according to the methods described above . The adsorption filtrate was also assayed.

Booher (1934) reported that adsorption on Lloyd's reagent of a 40 - 50 fold concentrate of vitamin B_2 prepared from whey and subsequent elution with a mixture of four parts of water, one part of pyridine and one part of methyl alcohol gave a highly active concentrate of the vitamin.

OUTLINE OF FEEDING EXPERIMENTS

A. 60% Alcohol Extract of Marmite, adsorbed with Fullers' earth.

Group XXIV Control Diet heated at 105°-120°C. + the eluate in an amount equivalent 6% Marmite. After 2nd week eluate increased to amount equivalent to 12% Marmite.

B. 80% Alcohol Extract of Marmite

I. Adsorbed on Fullers' Earth

Group XXV Control Diet heated at 105°-120°C.
1st and 2nd week + eluate equivalent to 10% Marmite
3rd week + eluate equivalent to 20% Marmite
4th week + eluate equivalent to 25% Marmite

Group XXVI Control Diet heated at 96°-110°C. + eluate equivalent to 12% Marmite.

Group XXVII Control Diet heated at 96°-110°C. + adsorption filtrate equivalent to 12% Marmite

II. Adsorbed on Lloyd's reagent

Group XXVIII Control Diet heated at 96°-110°C. + eluate equivalent to 12% Marmite.

Group XXIX Control Diet heated at 96°-110°C. + adsorption filtrate equivalent to 12% Marmite.

RESULTS

The experimental results are assembled in Table IV and the growth rates graphed in Figs. IX, X and XI.

NOTES

A. 60% Alcohol extract of Marmite adsorbed on Fullers' Earth.

Group XXIV Control Diet heated at 105°-120°C. + the eluate equivalent to 6% Marmite. After 2nd week eluate increased to an amount equivalent to 12% Marmite.

White Plymouth Rock Chicks. The average weights of these chicks were slightly lower than those fed the heated control (Group IV) alone. The gain in weight at three weeks was 8 gms. while with the heated control it was 10 gms. The mortality was 100%. At the end of the 3rd week all the chicks were dead. On the heated control diet the chicks were all dead at the end of the 4th week. There was one case of pellagra.

B. 80% Alcohol Extract of Marmite.

1. Adsorbed on Fullers' Earth

Group XXV Control Diet heated at 105°-120°C.
1st and 2nd week + eluate equivalent to 10% Marmite
3rd week + eluate equivalent to 20% Marmite
4th week + eluate equivalent to 25% Marmite.

Rhode Island Red Chicks. The average gain in weight in three weeks was 31 gms. as compared with 91 gms. for the same period with the same breed of chicks fed the same heated control supplemented with an 80% alcohol extract of Marmite equivalent to 10% Marmite. The mortality was 100%. There were three cases of pellagra.

Group XXVI Control Diet heated at 96°-110°C. + eluate equivalent to 12% Marmite.

Rhode Island Red Chicks. The average gain in weight for three weeks was 39 gms. as compared with 100 gms. when the same heated control supplemented with an 80% alcohol extract of Marmite equivalent to 8% Marmite was fed. This means that the eluate contains 25% of the growth promoting activity of the alcohol extract. There were six cases of pellagra but no mortality by the end of the 4th week.

Group XXVII Control Diet heated at 96°-110°C. + adsorption filtrate equivalent to 12% Marmite.

Rhode Island Red Chicks. At three weeks the weight was the same as that when the unheated basal control was fed or when the

same heated control supplemented with an 80% alcohol extract of Marmite equivalent to 8% Marmite was fed. The rate of gain was different, however, as the weights at the end of the first and second week were greater than those of the two controls. The gain in weight in the 4th week was 67% of the gain in weight in the 3rd week. The same relationship in the gain of weight was obtained with the heated control supplemented with an 80% alcohol extract of Marmite equivalent to 8% Marmite. In this case the gain in weights in the 4th week was 68% of that in the 3rd week.

There was no pellagra, but there were cases of "paralysis", two in the second week showing symptoms similar to those described by Keenan et al. (1933) one was fatal and the other recovered. Another case occurred in the 3rd week with symptoms resembling those described by Halliday (1934) in rats. This chick recovered. In the 4th week there was one more case showing the Keenan (1933) syndrome.

II. Adsorbed on Lloyd's Reagent

Group XVIII Control Diet heated at 96°-110°C. + the eluate equivalent to 12% Marmite.

Rhode Island Reds. The average weights are practically the same as those of chicks fed the eluate from Fullers' earth. The incidence of pellagra was also the same, but the mortality occurs earlier and was twice that which resulted on feeding the eluant from Fullers' earth. In this group there was greater variation in weight and in the severity of pellagra among the individual chicks.

Group XIX Control Diet heated at 96°-110°C. the adsorption filtrate equivalent to 12% Marmite.

Rhode Island Reds. The weights of these chicks are much higher than those receiving either the unheated basal control or the same heated control supplemented with an 80% alcohol extract of Marmite equivalent to 8% Marmite, or those fed the adsorption filtrate from Fullers' earth equivalent to 12% Marmite. The weight at the end of the first week more than doubled the average initial weight of 32.5 gms. With the basal unheated diets the average gain is about 20 grams in the first week. The evidence is therefore that three quarters of the potency of the 80% alcohol extract of Marmite remained in the adsorption filtrate from Lloyd's reagent. There was one death in the 2nd week but no pathological symptoms appeared up to the middle of the 4th week. There were however indications that the rate of growth was declining.

COMMENTS ON RESULTS

An eluate from a Fullers' earth adsorption of a 60% alcohol extract of Marmite did not show any vitamin B_2 activity in amounts

equivalent to 6% and 12% Marmite when added to a control diet heated at 105°-120°C. The extract appeared to aggravate the deficiency symptoms. This is indicated by the higher mortality at an earlier age. Later experiments show that only one-quarter or at the most one-third of the vitamin B_2 in an alcohol extract of Marmite was adsorbed on Fullers'earth. An amount of the extract equivalent to 10% Marmite was necessary to prevent pellagra when supplementing the diet heated at 105°-120°C. These findings account for the inactivity of the eluate when fed in amounts equivalent to 6% Marmite. They do not, however, explain the increased severity of the symptoms. On the same basis we can also account for the slight activity of an eluate from a Fullers" earth adsorption of an 80% alcohol extract of Marmite added to the same heated control as above in successive amounts equivalent to 10%, 20% and 25% of Marmite.

The results obtained with Group XXVII show that the growth promoting activity of an amount of the adsorption filtrate equivalent to 12% Marmite is roughly the same as that given by an amount of 80% alcohol extract of Marmite equivalent to 8% Marmite. We may conclude therefore that two thirds of the growth factor contained in the 80% alcohol extract of Marmite was not adsorbed on Fullers' earth but remained in the adsorption filtrate. As we have previously stated (notes on Group XXVI) the eluate contained one fourth of the activity of the 80% alcohol extract of Marmite. Eleven-twelfths of the original activity of the 80% alcohol extract is thus accounted for. One twelfth of the original activity was not eluted from the adsorbate or was lost during the washing of the adsorbate free from chloride.

We noticed that on prolonged washing of the Fullers' earth adsorbate it became exceedingly difficult to effect its separation by centrifuging and some loss in the supernatant resulted. Lepkovsky (1935) found that about one half of the vitamin B_2 potency of liver extracts was lost in the first adsorption with Fullers' earth.

While we cannot be so definite in explaining the behaviour of the alcohol extract of Marmite adsorbed on Lloyd's reagent it would appear that three-quarters of the activity remained in the adsorption filtrate and one quarter was adsorbed. This indicates that Lloyd's reagent adsorbed less vitamin B_2 than did Fullers' earth. However the total vitamin B_2 content of the alcoholic extract was accounted for more quantitatively in the two fractions (adsorbate and adsorption filtrate) when Lloyd's reagent was used than when Fullers' earth was employed.

Elvehjem and Koehn (1933) found that the material in liver extract adsorbable on Fullers' earth was inactive when tested with avitaminosis B_2 chicks and that the adsorption filtrate was highly active.

FIFTH SERIES

THE CURATIVE METHOD OF ASSAYING VITAMIN B_2

So far all assays except one (Group XV) have been preventative. A preliminary experiment was carried out to observe the effect on chicks of the curative method.

OUTLINE OF EXPERIMENT

Nine chicks were fed for five weeks the control diet heated at 95°-100°C. At the end of this period all the chicks showed subnormal growth and five had pellagra. They were now divided into three groups as follows:

Group II Control diet heated at 95°-100°C.

Group B Changed to the unheated basal.

Group C Control diet heated at 95°-100°C. + 4% Marmite.

These groups were fairly uniform in weight at the beginning of the fortnightly experiment and each contained one or two of the five cases of pellagra.

RESULTS

The results are graphed in Fig. II B. and they are summarized in the following table:

Weeks	Group II			Group B			Group C		
	Wt. in Grams	Pell-agra	Fort-ality	Wt. in Grams	Pell-agra	Fort-ality	Wt. in Grams	Pell-agra	Fort-ality
5	149	1		181	2		154	2	
6	181	1		215	+2		195	+2	
7	207	1		276			254		
		100%							

NOTES

Group II The average weights of the chicks continued subnormal and all the chicks had pellagra at the end of the experiment. There was no mortality.

Group B The average gain in weight of these chicks during two weeks was 114 gms. as compared with the 58 gms. gain in weight of the chicks in group II during the same period. The chicks with pellagra recovered in the sixth week.

Group C These chicks gained 110 gms. during the two experimental weeks. The chicks suffering from pellagra recovered in the sixth week.

SUMMARY OF RESULTS

Chicks fed a control diet heated at 95°-100°C. showed retarded growth and a 50% incidence of pellagra at the end of the fifth week. When the diet was changed to the unheated control or the heated control was supplemented with 4% Marmite the gain in weight during two weeks was about twice that of the chicks which were continued on the heated control. The chicks changed to the unheated control diet or given the 4% Marmite supplement were cured of pellagra a week after the change in diets occurred.

GENERAL DISCUSSION

The vitamin B_2 requirements of the chick appear to be relatively large since they failed to grow normally and developed, after four weeks, a characteristic pellagrous dermatitis, when fed a diet the vitamin B_2 content of which was only partially destroyed by heating. The chick is therefore a particularly useful experimental animal for vitamin B_2 studies.

The basal diet used in these experiments was adequate for normal growth and development. Dry heat treatment of the same diet destroyed several essential dietary factors. The nature and severity of the pathological disturbances exhibited by chicks fed the various heat treated diets was related to the actual temperatures within the range of 95°-130°C. to which the diet had been subjected. It is evident from our results that, of the thermolabile substances contained in the diet vitamin B_2 is theleast stable in dry heat, and that it should be possible to find a temperature or range of temperature to which to expose the dry diet so that the maximum destruction of vitamin B_2 is obtained without significantly affecting other essential factors.

A careful examination of the symptoms shown by the birds fed the diets heated at various temperatures and particularly in relation to their rate of growth, affords information which is of a very considerable assistance in establishing the optimum temperatures at which vitamin B_2 alone is destroyed. In our experiments we found that chicks fed a diet heated dry at 100°C. for six days developed symptoms of vitamin B_2 deficiency only. These symptoms however were not sufficiently drastic to serve

our purpose. Heating at 110°, 120° and 150°C. as well as storing for six months the diet heated at 100°C. destroyed a number of factors other than vitamin B_2 and the symptoms of their deficiency complicated the vitamin B_2 assays. The chief deficiency symptoms other than pellagra were haemorrhages, and paralysis. When the diets heated at 100°C. and stored, and at 120°C. were fed alone growth was retarded and the only symptoms were those of pellagra. Among the birds receiving the unsupplemented diet heated at 110°C. there first developed a case of polyneuritis, then one of pellagra and polyneuritis and one of paralysis. Of the six chicks with pellagra, two recovered spontaneously. The experience with this diet indicates that whenever there is a deficiency of the entire vitamin B complex with its resultant mortality the only apparent symptoms are those of pellagra. Perhaps the requirement of the chick for the growth promoting pellagra preventing factor is greater than for any of the other components of the vitamin B complex. Heating at 110°C. may be in effect a borderline temperature where the destruction of the different vitamin B factors is evenly balanced and we must look for our differential temperature somewhere below 110°C. and above 100°C.

With the one exception cited above, paralysis occurs only in the absence of pellagra. Evidence is presented for the existence of three types of paralysis. In the one type the chicks could only get up with great difficulty, they would take a few steps when roused but preferred to squat. This type was usually fatal probably owing to the inability of the birds to reach food and water. These symptoms occurred in birds fed the diet

heated at 100°C. and stored for six months when either supplemented with 6% Marmite or with an alcohol extract equivalent to 6% Marmite, and when fed the diet heated at 96°-110°C. supplemented with the adsorption filtrate from fullers' earth equivalent to 12% Marmite. In all these cases growth was extremely rapid.

The birds with the second type of paralysis squatted also but tried to get up. This was usually accompanied by turning round and round and falling over to one side. These symptoms occurred on the same diets as above where the rate of growth was greatly accellerated. They showed improvement after Harris yeast was administered.

The third type of paralysis occurred only on feeding the diet heated at 96°-110°C. supplemented with 6% Marmite. The growth was not accelerated. The symptoms were very peculiar. The birds were squatting and when they tried to walk they fell with head bent downward. As the disease progressed they were not able to lift their heads, but squatted leaning on their heads. These cases were always fatal.

On the diet heated at 96°-110°C. there were two well defined groups those with pellagra and those with paralysis. Practically all the birds fed the eluates from alcoholic extracts of Marmite developed pellagra and showed retarded growth.

The chicks receiving the adsorption filtrate, or 6% Marmite or the 80% alcohol extract equivalent to 8% Marmite showed a 50% incidence of paralysis preceded by average or better than average growth. In every one of these groups there is a sudden drop in the rate of growth at the end of the third week. As we

mentioned before the chicks receiving the heated diet alone had pellagra, polyneuritis and paralysis and their weights showed the same characteristic drop at the end of the fourth week. There is no doubt that this drop in weight was closely associated with the occurrence of paralysis.

The great incidence of paralysis among these chicks suggested the possibility of an epidemic or of some toxic substance in the diet. However when we changed the diet of the chicks receiving the Lloyd's reagent adsorption filtrate to an unheated basal diet the chicks did not develop paralysis.

There was never a case of paralysis among chicks receiving the control diet heated at 105°-120°C. whether supplemented or not. The peculiarity of this diet, however, was that though the supplement of Marmite or its alcohol extract was raised sufficiently to prevent pellagra the weights of the chicks could not be increased beyond a certain point, as if there still existed a deficiency of another growth factor which is not contained in Marmite. The absence of paralysis where growth is retarded and its great incidence where growth is accelerated indicates that the requirement for a growth factor varies with the rate of growth of the chick. Booher (1934) came to the same conclusion in her experiments with rats. The factor whose insufficiency precipitates paralysis is evidently also a growth factor because the sudden drop in weight and the pathological symptoms are synchronous.

The pellagra preventing factor is also closely bound up with a growth factor. The pellagrous dermatitis appears to be the final visible expression either of a long continual slight

insufficiency of a growth factor or an acute shortage thereof. Pellagra appears earlier when the deficiency is acute and later when the growth is only slightly subnormal. The growth is always retarded when there are symptoms of pellagra. Whenever enough of the anti-pellagra factor is present there is also better growth. Itter, Orent, McCallum (1935) found that there is an apparent lowering of the glutathione in the blood and liver of rats deprived of vitamin B_2 which can be appreciably raised by addition of 5% whole yeast to the diet. The administration of glutathione and cysteine to rats depleted of the vitamin B_2 complex resulted in maintenance of weight over a period of six weeks, whereas the controls showed a loss of weight. The possible role of vitamin B_2 in sulphur metabolism gains additional interest in view of the most recently postulated dual nature of vitamin B_2. Vitamin B_2 according to Gyorgy (1935) is a complex composed of lactoflavin and the pellagra-preventing factor. Harris (1935) claims that flavin has no antipellagra action. It is however a constituent of the vitamin B_2 complex in the sense that it causes some improvement in the weight curve when given as a supplement to vitamin B_1. With vitamin B_1 plus the pellagra-preventing fraction only, pellagra was always prevented or cured. Without further addition of flavin, however, body weights remained subnormal. The combined effect of vitamin B_1 plus flavin plus Peters' eluate (containing the antipellagra factor) was approximately equal, in its weight-promoting action and in the cure or prevention of symptoms, to that of the total complex given in the form of Marmite.

The findings of Gyorgy and Harris, as well as the claim of Kuhn (1933) that flavin is adsorbed on Fullers' earth and the counterclaim of Elvehjem and Koehn (1935) that the factor promoting growth and preventing pellagra in chicks was not adsorbed on Fullers'earth but remained in the adsorption filtrate, are illustrated to some extent by our results with the adsorption of alcohol extracts of Marmite on Fullers' earth and Lloyd's reagent, summarized in Table IV. Our eluates which presumably contain flavin did not prevent pellagra and gave retarded growth. The adsorption filtrates prevented pellagra and gave good growth for three weeks, but showed a subsequent drop in the rate of growth. However Marmite (6%) or an alcohol extract of Marmite equivalent to 8% Marmite showed the same sudden decline in the 4th week. Therefore in our experiments the decline in weight is explainable not only as due to the splitting of the vitamin B_2 complex into flavin and the pellagra preventing substance, which though both growth promoting can only promote normal growth when given together, but that the heating of the diet at 110°C. destroys a growth factor which is needed by chicks in increasing amounts as the growth progresses.

SUMMARY

1. The chick has been found to be a particularly useful animal for vitamin B_2 studies.
2. There is a definite quantitative relationship between the rate of growth of chicks and the vitamin B_2 content of the diet.
3. A storage of vitamin B_2 in the body of the chick is indicated.
4. A diet of natural foodstuffs can be rendered deficient in vitamin B_2 when subjected to dry heat treatment at 100°C. or above for six days. Using this diet variously supplemented the following observations were made.
   - A. When added in sufficient amounts Marmite will adequately supplement a vitamin B_2 deficient diet.
   - B. Storing a diet which has been heat treated increases the destruction of vitamin B_2, but also causes the destruction of other essential factors.
   - C. Vitamin B_2 is shown to be completely soluble in either 60% or 80% alcohol. The 80% alcohol extraction effects a greater purification of the active substance.
   - D. The vitamin B_2 content of alcohol extracts of Marmite is only partly adsorbed on fullers' earth, the major part of the activity remains in the adsorption filtrate.
   - E. Lloyd's reagent adsorbs less vitamin B_2 from alcohol extracts of Marmite than does fullers' earth.
5. An improved method for extracting Marmite with alcohol is presented. The preliminary fractionation of Marmite with alcohol has been improved by a separate fractionation with

water and alcohol. This procedure greatly simplifies the preparation of alcohol extracts of Jarmite and effects a quantitative extraction of the pigment.

TABLES AND GRAPHS

Table I

Showing the weekly increase in weight, incidence of pellagra, mortality and weekly food consumption of chicks fed on the control diet, and on the control diet heat treated.

Group	I A	I B	I C	II	III	IV	V
	Control			Control Heated			
	Leghorns	Fly. W.	R.I. Red	95-100°	95-100°	105-110°	95-100°
No. of chicks	10	8	10	10	7	8	7
Time in Weeks	Body weight in Grams						
1	38	32	33	34	44	47	38
2	75	78	84	72	58	51	57
3	105	118	130	96	76	57	81
4	142	168	188	122	100	70	92
5	192	248	253	151			
6		357	317				
Time in Weeks	Incidence of Pellagra						
1							
2					3	5	3
3					1		2
4				8			-2
5				2			
6							
% Pellagra				64%	57%	50%	
Time in Weeks	Mortality						
1				1	1	3	
2					2		2
3					2	1	1
4					2	2	1
5				1			
6							
% Mortality				13%	100%	100%	
Time in Weeks	Food Consumption (grams) per Chick						
1	56	41	34	33	26	29	33
2	85	76	67	54	48	56	45
3	71	131	99	65	53	64	48
4	170	175	142		125	125	5.
5	221	224	167	179			
6			222				
Total	561	647	752	351	262	304	108

* Stored for six months after heating.

Table II

Summarizing the rates of growth, evidences of dietary deficiency, mortality and food consumption of chicks fed on the various control (heated) diets supplementing with varying amounts of Yarmite.

Group	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
No. of Chicks	16	16	10	7	8	8	8	11	7
Time in weeks	Body Weight in Grams								
1	55	56	52	60	60	48	52	51	53
2	85	87	84	86	81	80	66	68	82
3	117	130	165	118	112	82	87	94	100
4	166	197	258	125	135	107	141	148	156
5	239	275	307	151	156		189	201	
6			407	181	177				
Time in Weeks	Incidence of Pellagra, Hæmorrhage and Paralysis								
	PH Pa	PH Pa	PH Pa	PH Pa	PH Pa	PH Pa	PH Pa	PH Pa	PH Pa
1									
2			4				1	6	
3					1 2	2	1	1	
4				1	1	1	2	3	1
5				1				2	
6					1				
Time in Weeks	Mortality								
1			2	1		5			
2	1		7				3		
3				1	2	2			
4				1			5	2	1
5				1	1	1			
6									
% Mortality	6%		90%	57%	50%	100%	71%	18%	
	Food Consumption (Grams) per Chick								
1	52	54	50	51	49	26	36	36	37
2	68	73	65	73	62	30	52	52	70
3	80	101		100	94	24	60	75	74
4				182	161	71	55	107	106
5	276	329		186	200		125	159	
6				250	245				
Total	476	557	115	822	851	181	286	409	287

Table III

Summarizing the rates of growth, evidences of dietary deficiency, mortality and food consumption of chicks fed on vitamin B_2 deficient diets supplemented with 80% alcohol or 80% alcohol extracts of Marmite

Group	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	XXIII
No. of Chicks	4	10	7	11	5	7	10	7	7
Time in Weeks	Body Weight in Grams								
1	53	55	59	58	46	55	55	55	57
2	73	85	87	84	67	88	79	78	84
3	98	133	120	119	80	97	108	115	128
4	127	197	129	132	113	136	158	152	157
5	153	287	150	148	163	139	205	206	
6	187	402	172	172	244	164			
7	197								
Time in Weeks	Incidence of Pellagra, Haemorrhage and Paralysis								
	PH Pa	PH Pa	PH Pa	PH Pa	PH Pa	PH Pa	PH Pa	PH Pa	PH Pa
1		1			1	2			
2		1			1	2			
3		1 2		1	1		2 2		
4	2	2	4	2	1	1	2 1		1
5	1				2	1	1		
6									
7	1								
Time in Weeks	Mortality								
1		1	1	4	2	2			
2		1	1	1			3		
3		2		2	1	2	1		
4			2			1	1		
5			1	1					
6									
7									
% Mortality	0	40%	71%	73%	60%	71%	50%	0	0
Time in Weeks	Food Consumption (grams) per Chick								
1	53	59	63	38	31	45	55	55	41
2	84	81	82	82	58	84	77	77	71
3	65	122	124	144	52	44	96	95	
4		214	217	164		84	161	119	102
5	179	250	201	113		155	160	121	
6	176	358	194	150		132			
7	208								
Total	715	1040	882	706		515	529	447	318

Table IV

Summarizing the rates of growth, evidences of dietary deficiency, mortality and food consumption of chicks fed on vitamin B_2 deficient diets supplemented with the material adsorbed from alcohol extracts of Marmite on Fullers' earth or on Lloyd's Reagent.

Group	XXIV	XXV	XXVI	XXVII	XXVIII	XXIX.
No. of Chicks	7	5	7	7	7	7
Time in Weeks	Body Weight in Grams					
1	42	52	67	65	59	69
2	49	61	75	99	73	110
3	50	67	85	127	82	131
4		85	99	146	103	
5						
6						
Time in Weeks	Incidence of Pellagra and Paralysis					
	Pel.Par.	Pel.Par.	Pel.Par.	Pel.Par.	Pel.Par.	Pel.Par.
1						
2	1	1	3	½	5	
3		3	3	1-1	5	
4				2-1		
5						
6						
Time in Weeks	Mortality					
1	1	1				1
2	5	1		1		
3	1	1		1	1	
4		2	1		1	
5						
6						
% Mortality	100	100				
Time in Weeks	Food Consumption (grams) per Chick					
1	23	32	47	44	41	49
2	30	45	66	79	58	106
3		65	62	96	71	136
4		57	61	132	75	
5						
6						
Total	53	177	236	351	245	291

Fig. 1

SHOWING THE EFFECT OF VARIOUS HEAT TREATMENTS OF THE CONTROL DIET IN DESTROYING THE GROWTH PROMOTING AND PELLAGRA PREVENTING FACTOR

Group I A White Leghorn Chicks - Control diet

Group II White Leghorn Chicks - Control diet heated at 95°-100°C.

Group I B White Plymouth RocksChicks - Control diet

Group III White Plymouth Rock Chicks - Control diet heated at 95°-100°C. and stored six months

Group IV White Plymouth Rock Chicks - Control diet heated at 105°-120°C

Group I C Rhode Island Red Chicks - Control diet

Group V Rhode Island Red Chicks - Control diet heated 96°-110°C.

NOTE. The number of chicks suffering from pellagra is indicated on the growth curves.
The number of chicks in each group is given (in brackets) following the group number.

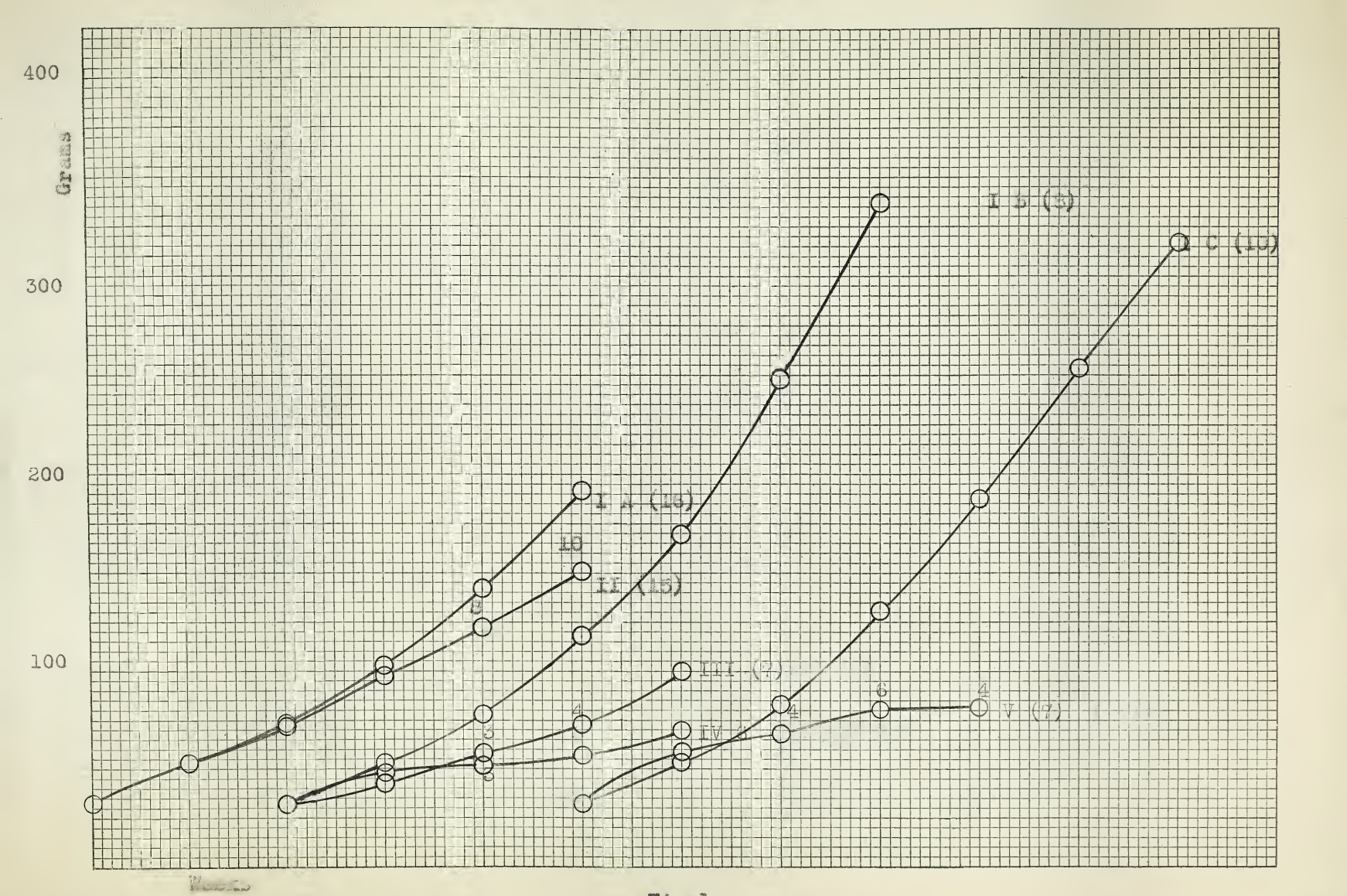


Fig.1

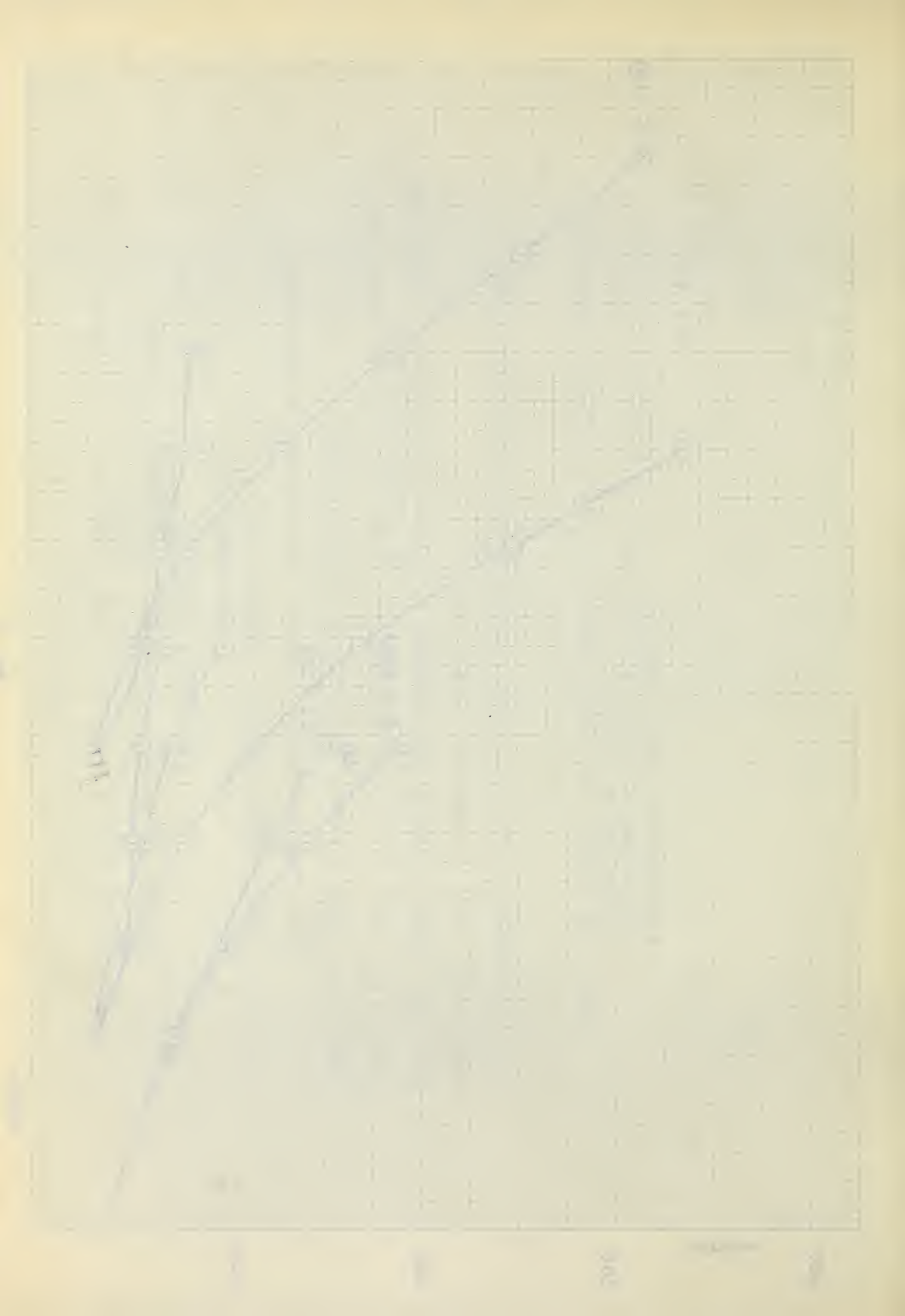


Fig. 2.

SHOWING THE INCREASED RATE OF GROWTH AND ABSENCE OF PELLAGRA WHEN MARMITE IS ADDED TO A HEATED CONTROL DIET.

A. Preventative assay.

Group I A Unheated control diet

Group II Control diet heated at 95°-100°C.

Group VI Control diet heated at 95°-100°C. + 2% Marmite

Group VII Control diet heated at 95°-100°C. + 4% Marmite

B. Curative assay

Showing the effect of supplementing with Marmite after growth has been retarded by feeding for five weeks the diet heated at 95°-100°C.

Group II Control diet heated at 95°-100°C.

Group B Changed to the unheated diet.

Group C Control diet heated at 95°-100°C. + 4% Marmite.

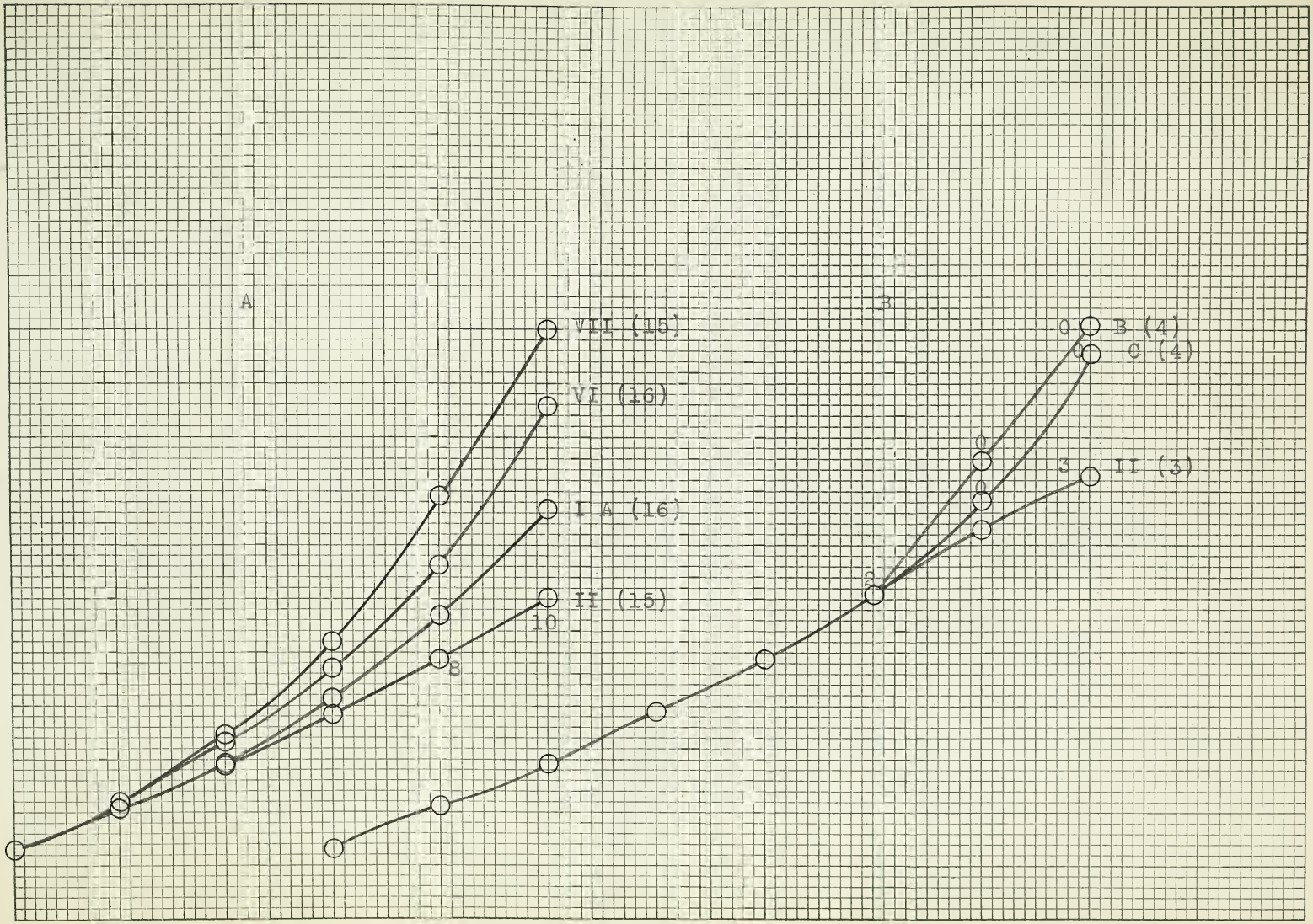


Fig. 2

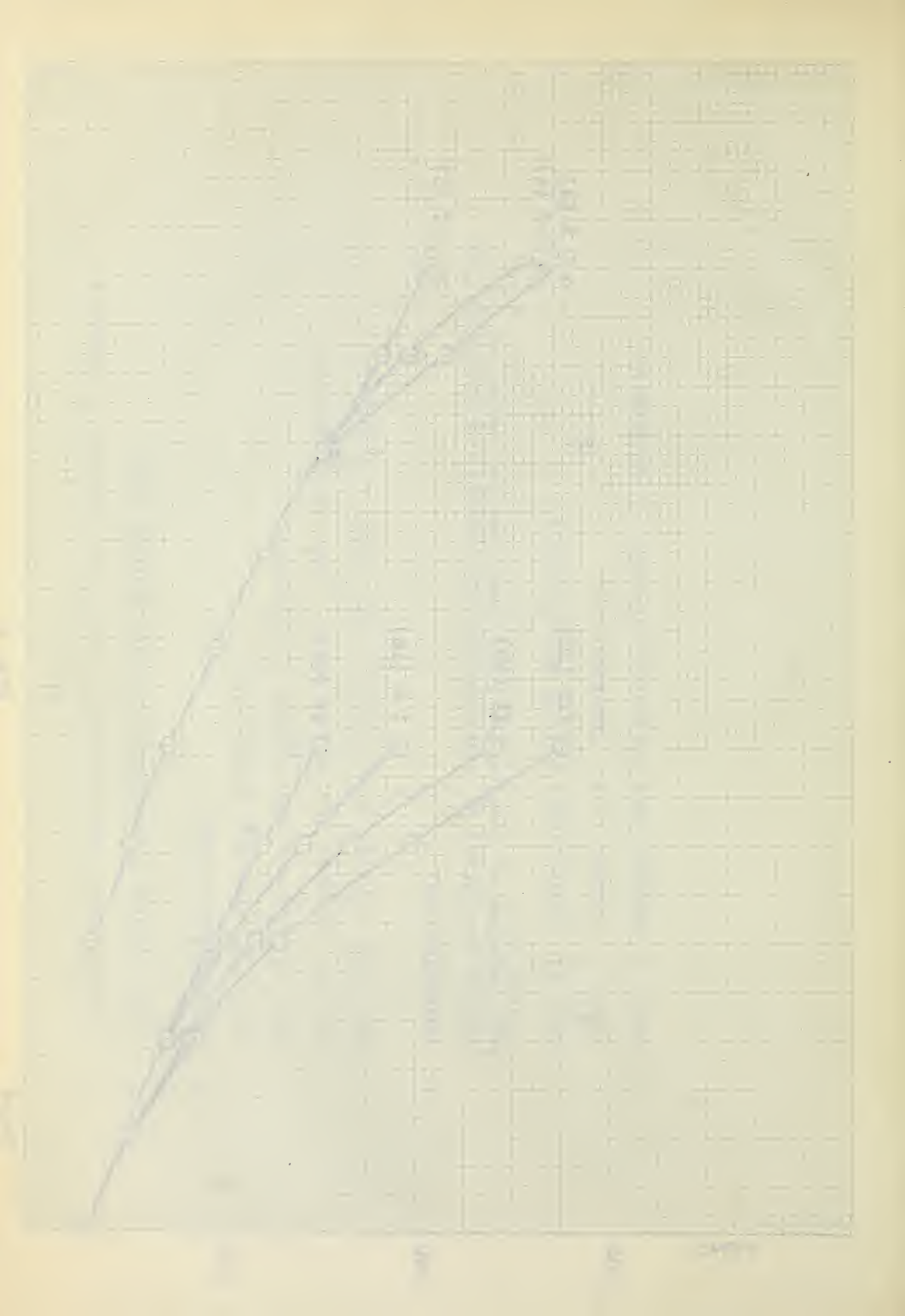


Fig. 3

SHOWING THE GROWTH RESPONSE AND ABSENCE OF PELLAGRA

WHEN MARMITE IS ADDED TO A HEATED CONTROL DIET

Group I B - Control diet

Group III - Control diet heated at 95°-100°C, stored six months

Group VIII - Heated control diet + 6% Marmite

- 1st and 2nd week - control diet heated at 95°-100°C. stored six months
- 3rd and 4th week - control diet heated at 130°C.
- 5th and 6th week - control diet heated at 105°-120°C.

Group IX - Above + 4% Marmite

Group X - Above + 6% Marmite

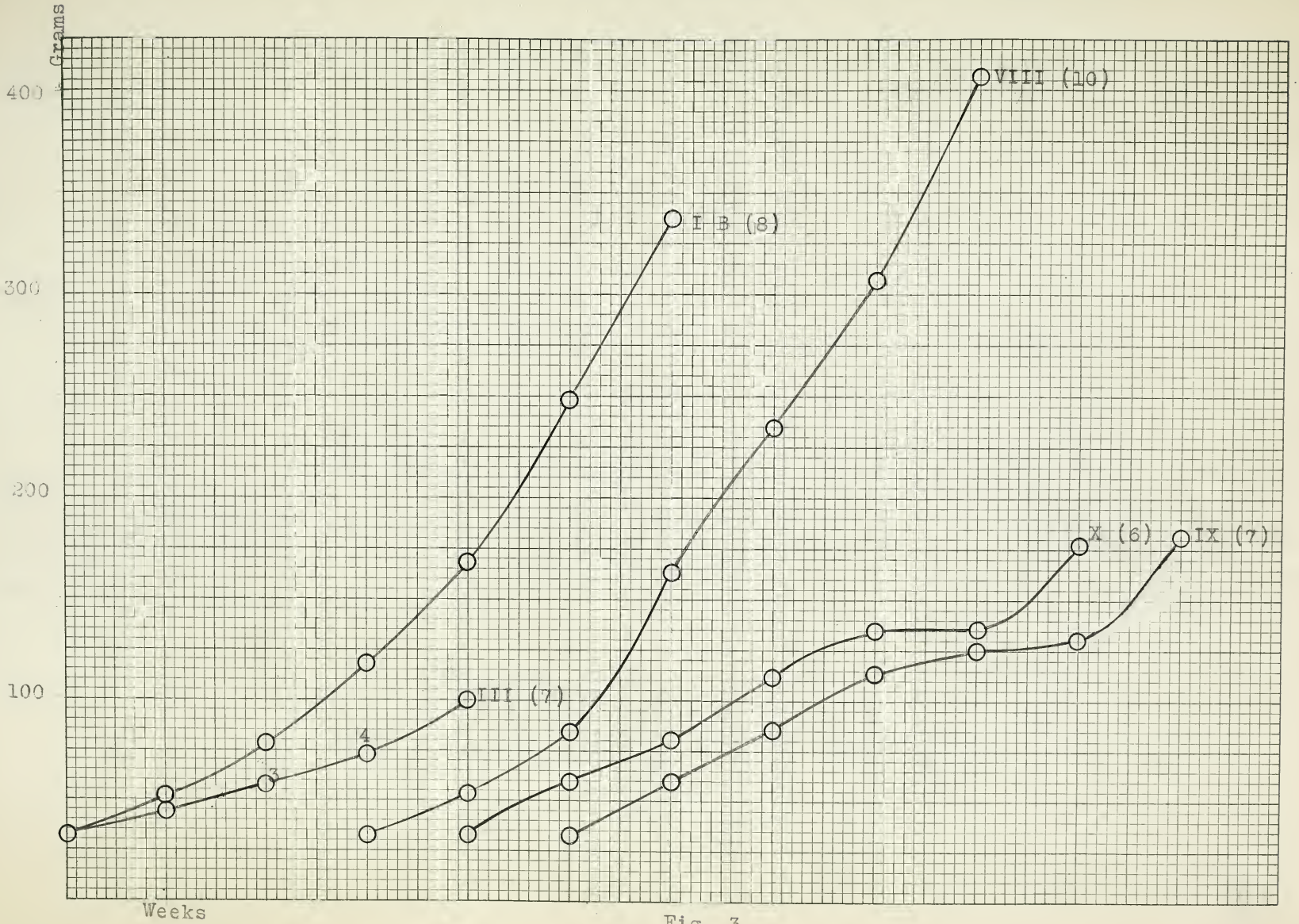


Fig. 3

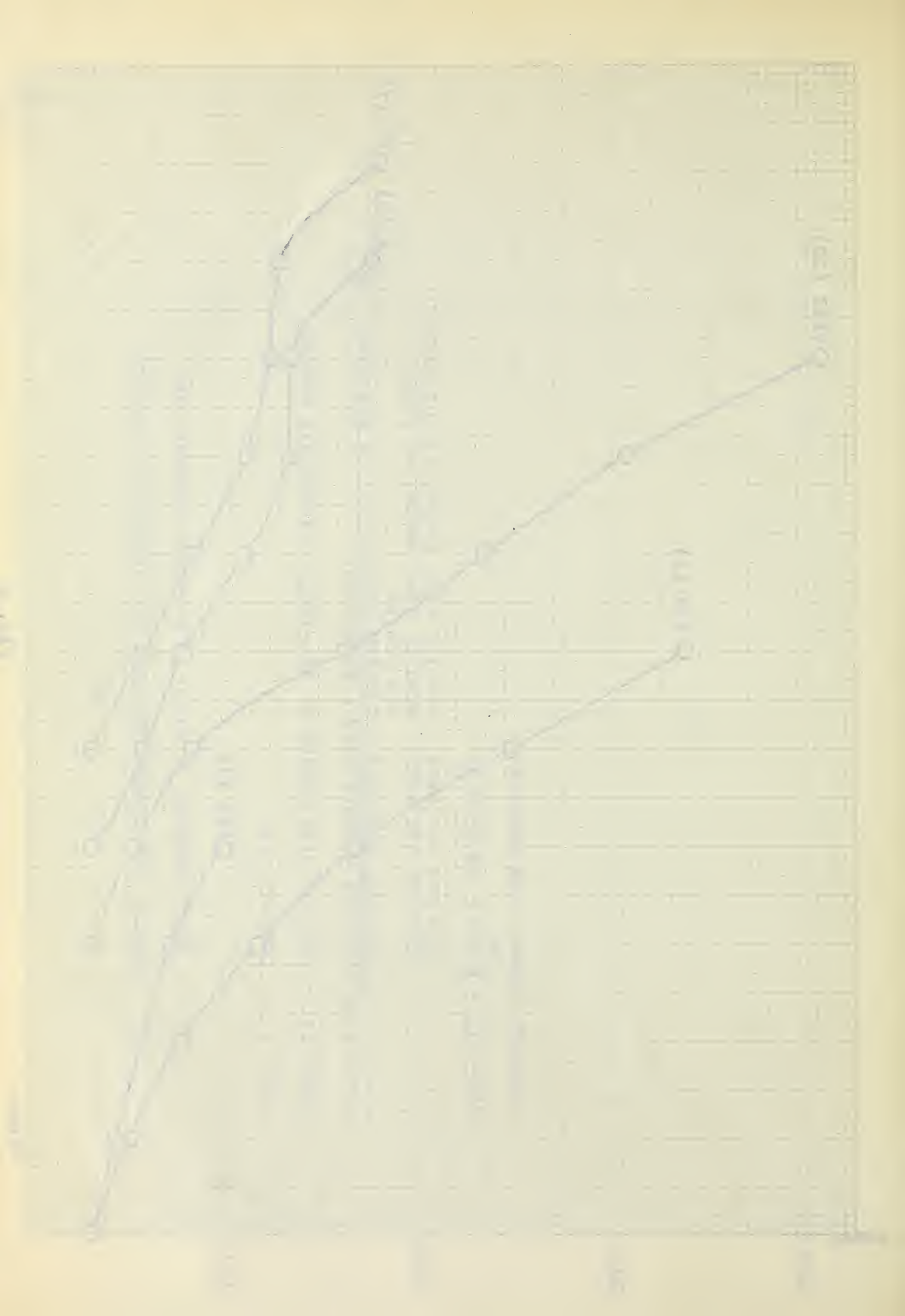


Fig. 4.

SHOWING THE EXTREME DEFICIENCY OF THE CONTROL DIET

HEATED AT 105°-120°C.

Group I B - control diet

Group IV - control diet heated at 105°-120°C.

Group XI - control diet heated at 105°-120°C.

1st and 2nd week - 3% Marmite added
3rd week - 4% Marmite added
4th week - 6% Marmite added

Group XII - control diet heated at 105°-120°C. + 6% Marmite

Group XII - control diet heated at 105°-120°C. + 6% Marmite for two weeks after two weeks + 8% Marmite.

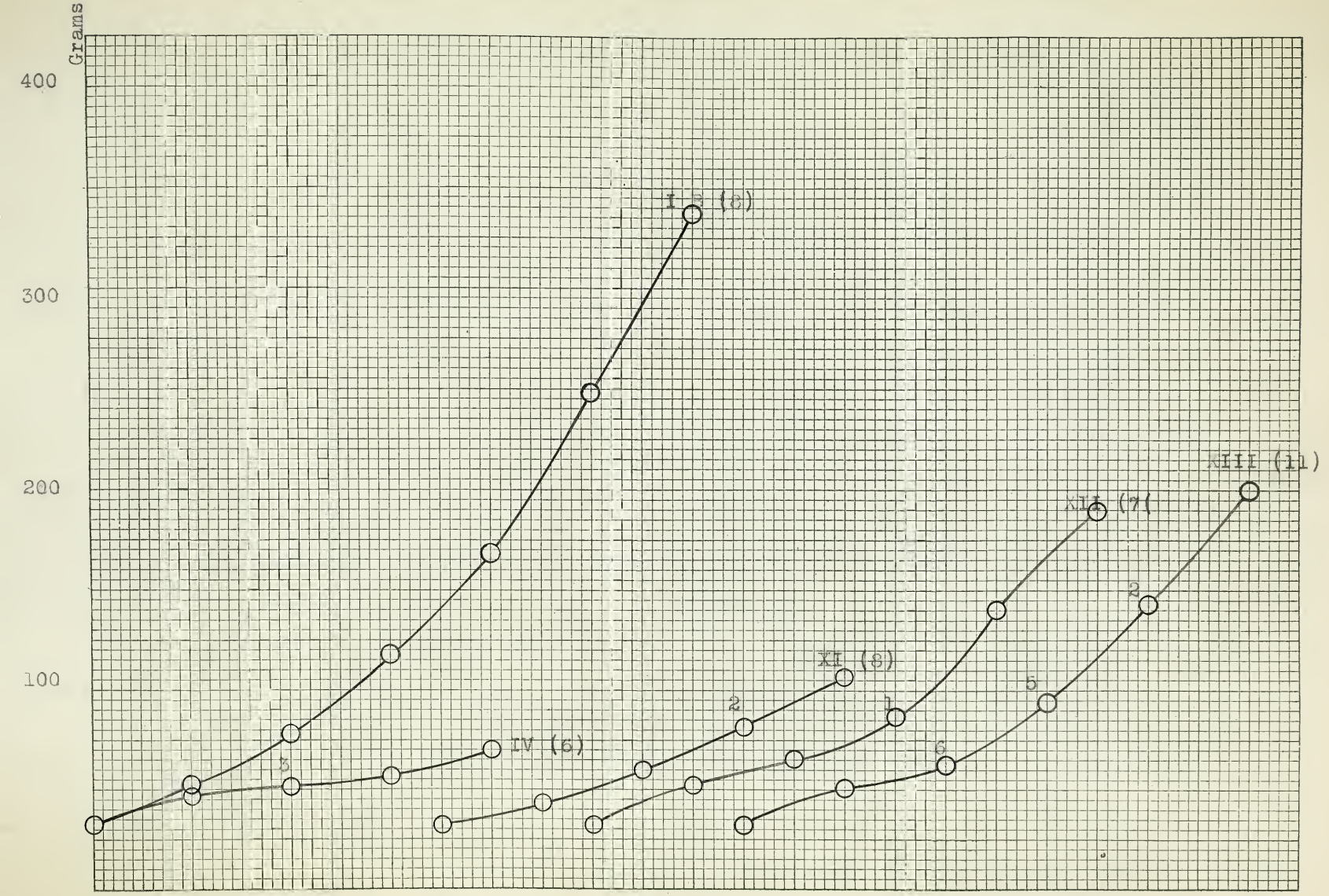


Fig. 4

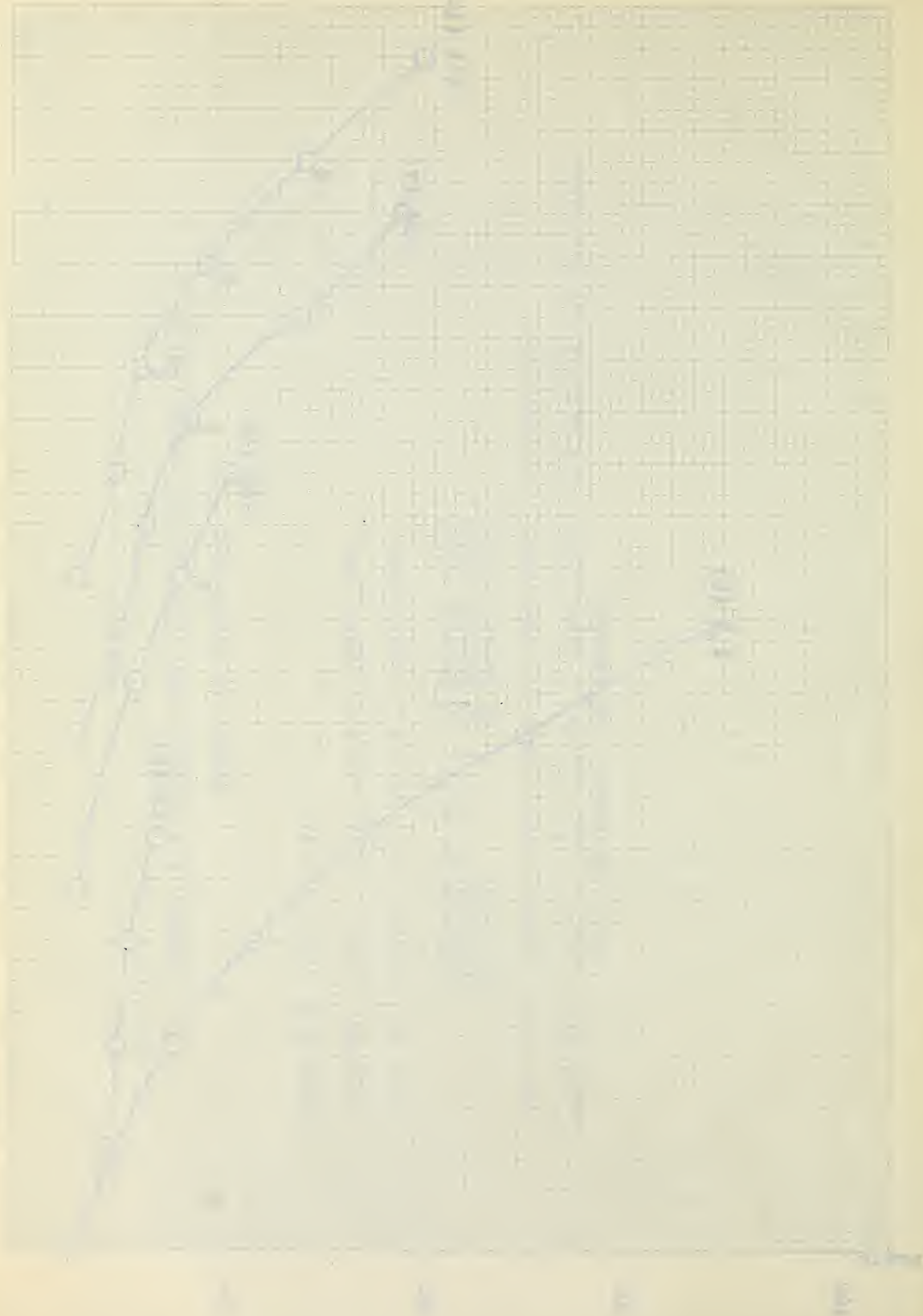


Fig. 5

SHOWING THE EFFECT OF SUPPLEMENTING THE

HEATED CONTROL DIET WITH MARMITE

Group I C - Control diet.

Group V - Control diet heated at 96°-110°C.

Group XIV - Control diet heated at 96°-110°C. + 6% Marmite

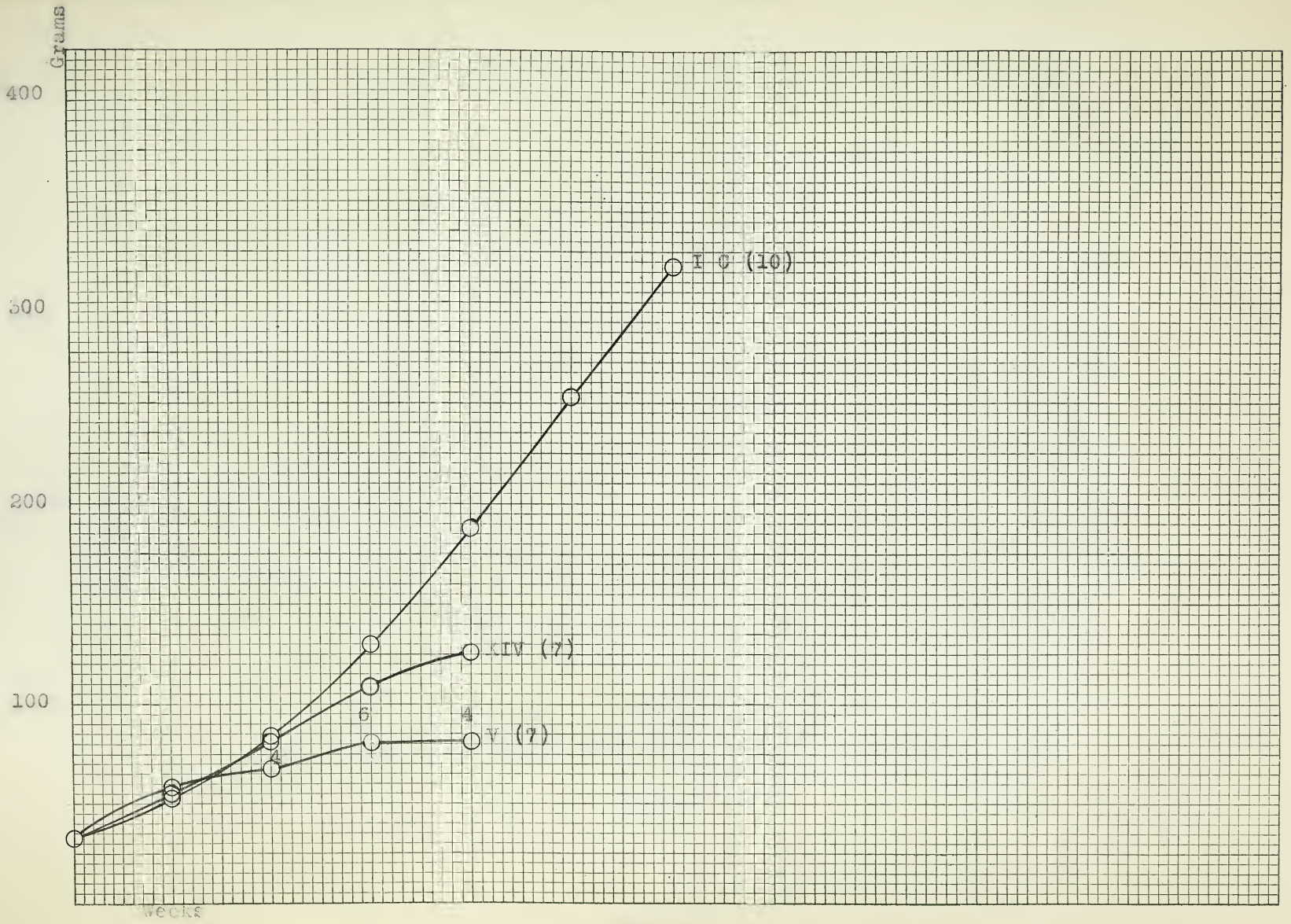


Fig. 5

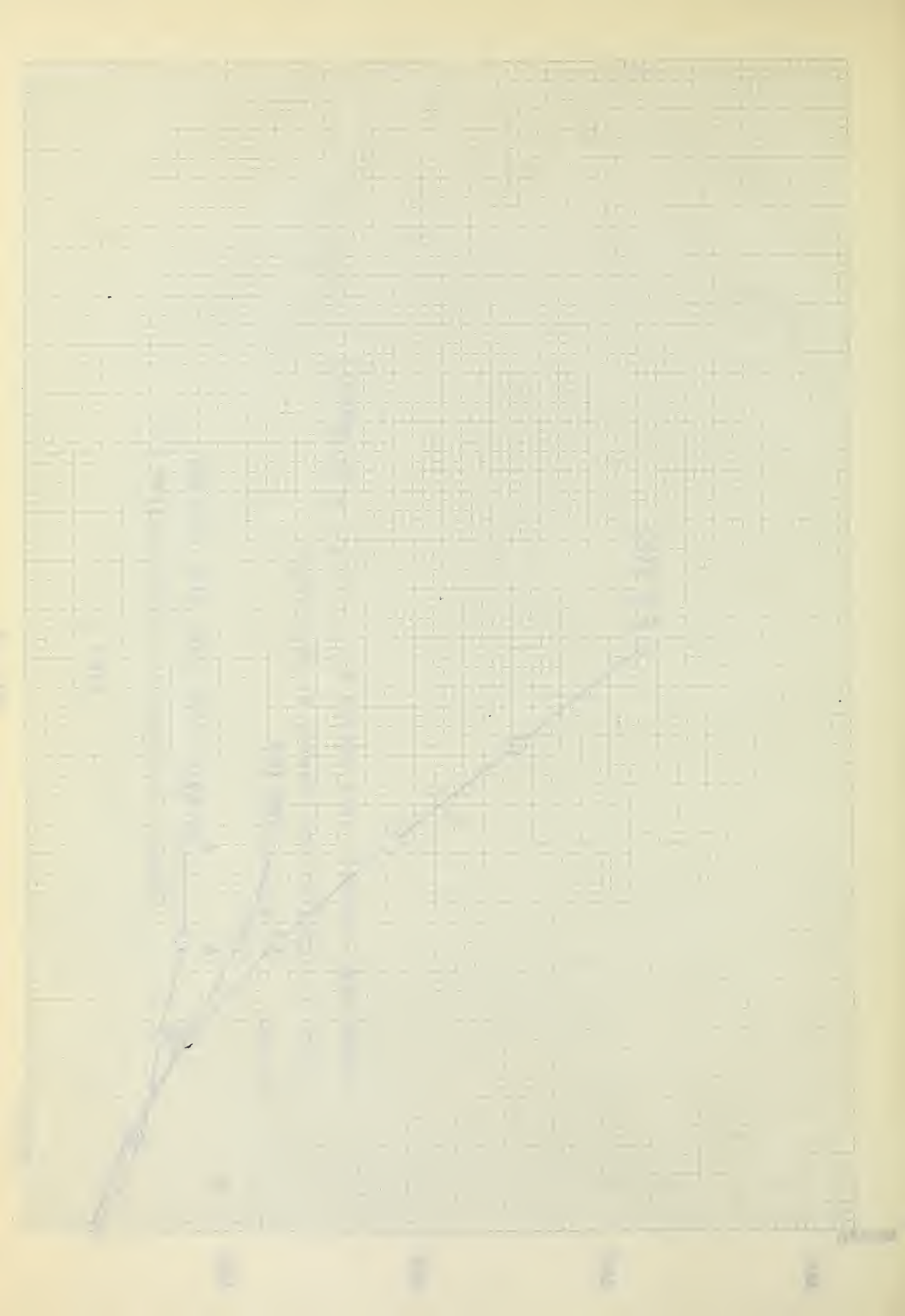


Fig. 6.

SHOWING THE COMPARATIVE GROWTH ~~RES~~ RESPONSE WITH

ALCOHOL EXTRACTS OF MARMITE

Group II - Control diet heated at 95°-100°C. fed for seven weeks.

Group XV - Control diet heated at 95°-100°C. supplemented in 6th and 7th week with an 80% alcohol extract of Marmite equivalent to 4% Marmite.

Group I B - Control diet

Group III - Control diet heated at 95°-100° C., stored six months.

Group VIII - Control diet heated at 95°-100°C. + 6% Marmite.

Group XVI - Control diet heated at 95°-100°C. + 60% alcohol extract of Marmite equivalent to 6% Marmite.

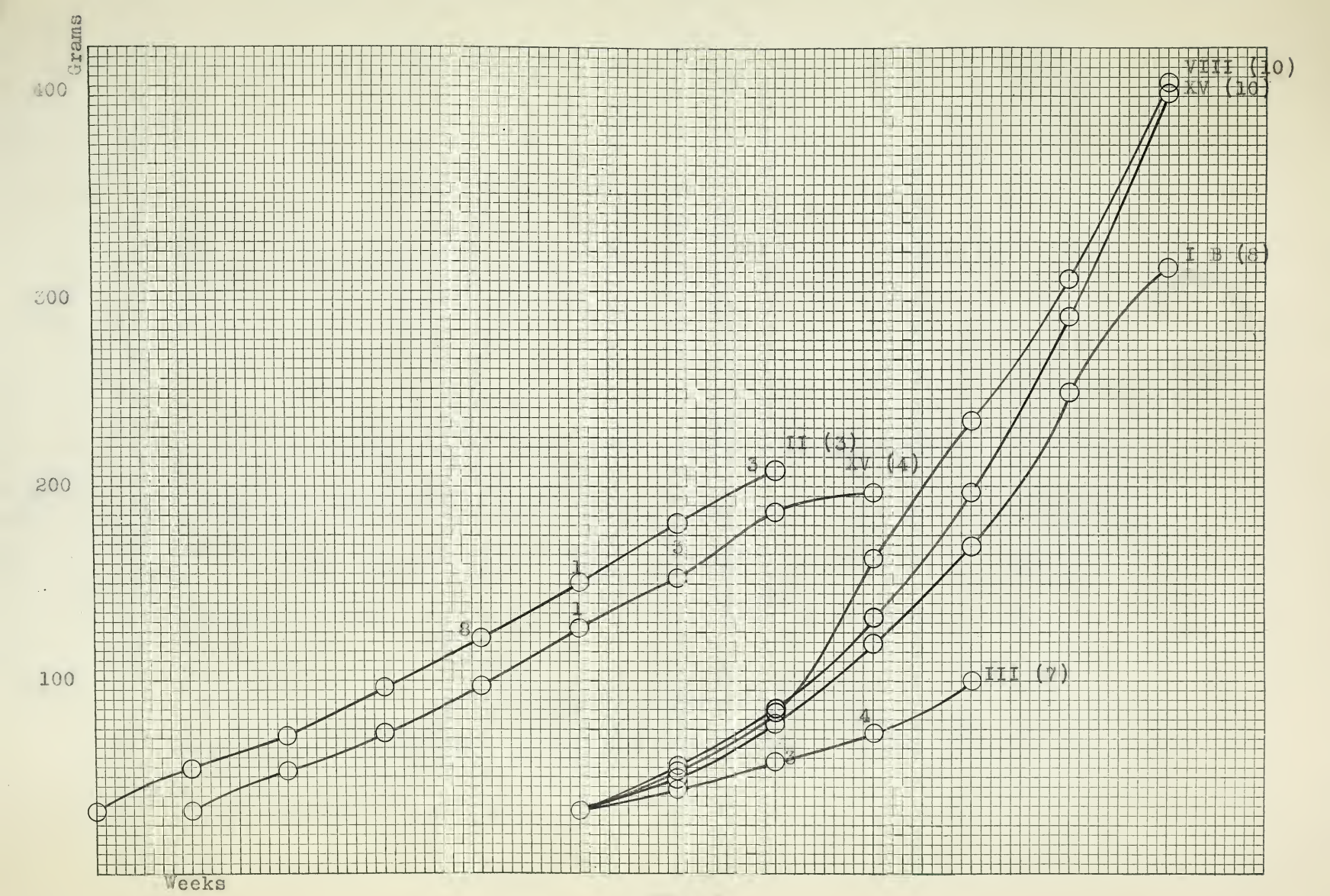

Grams
400
300
200
100
Weeks
VIII (10)
XV (10)
I B (8)
II (3)
IV (4)
III (7)
3
3
1
1
8
4
3

Fig. 6

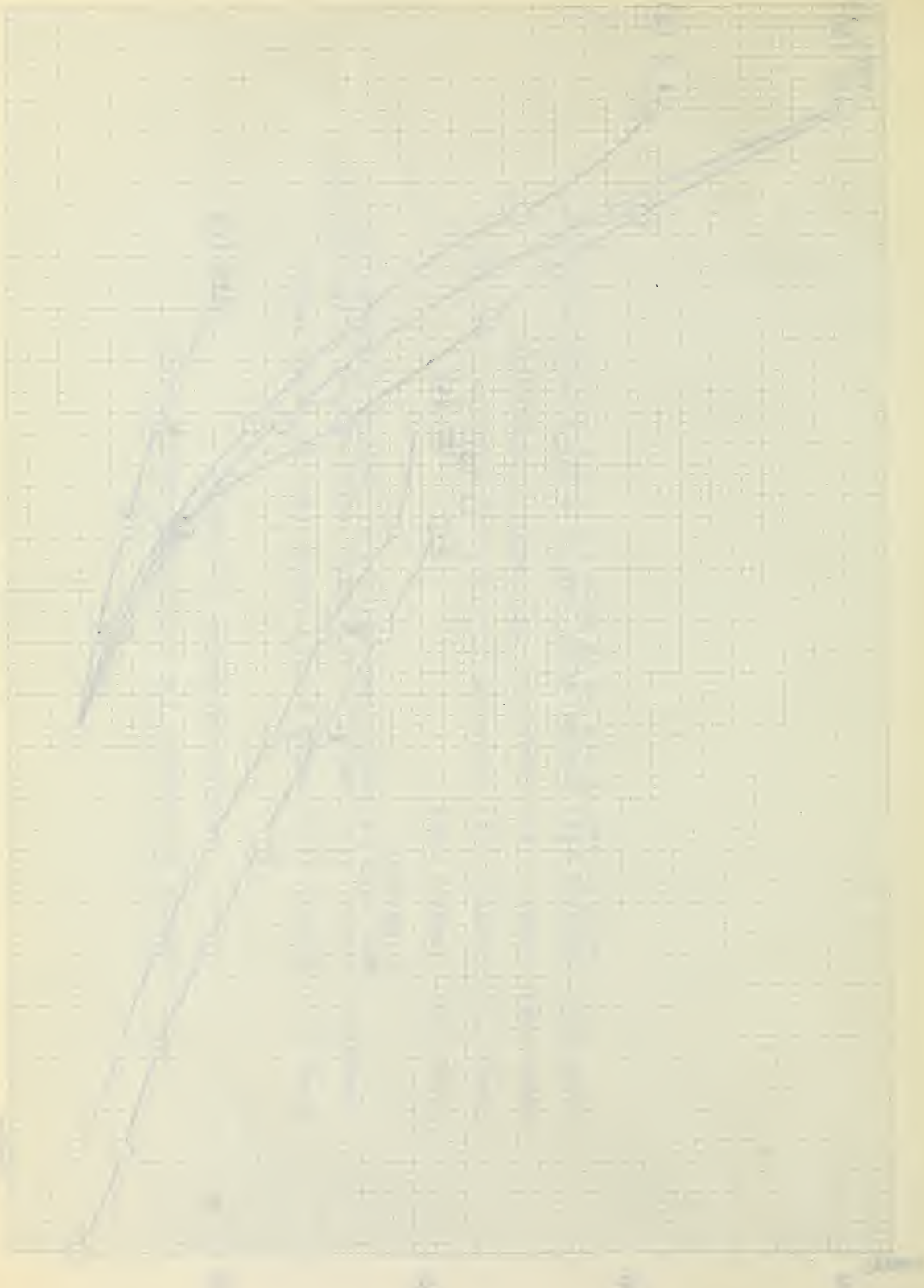


Fig. 7

ILLUSTRATING THE COMPLETE EXTRACTION OF VITAMIN B_2

FROM MARMITE BY 60% OR 80% ALCOHOL.

Group X - 1st and 2nd week - control diet heated at 95°-100°C., stored six months + 6% Marmite.
3rd and 4th week - control diet heated at 130°C. + 6% Marmite.
5th and 6th week - control diet heated at 105°-120°C. + 6% Marmite.

Group XVI - Same control diets + 60% alcohol extract of Marmite equivalent to 6% Marmite.

Group XVII - Same control diets + 80% alcohol extract of Marmite equivalent to 6% Marmite.

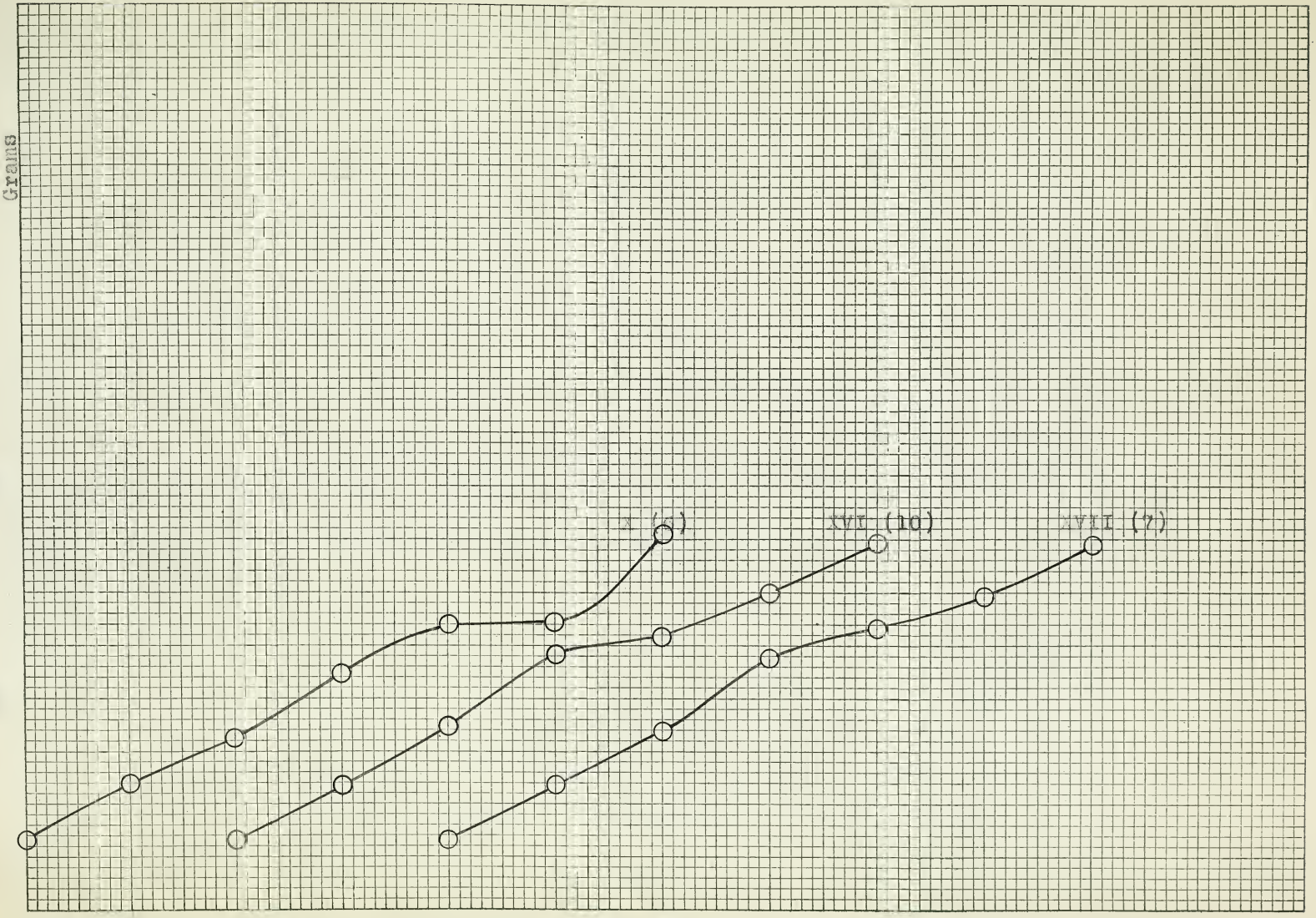


Fig. 7

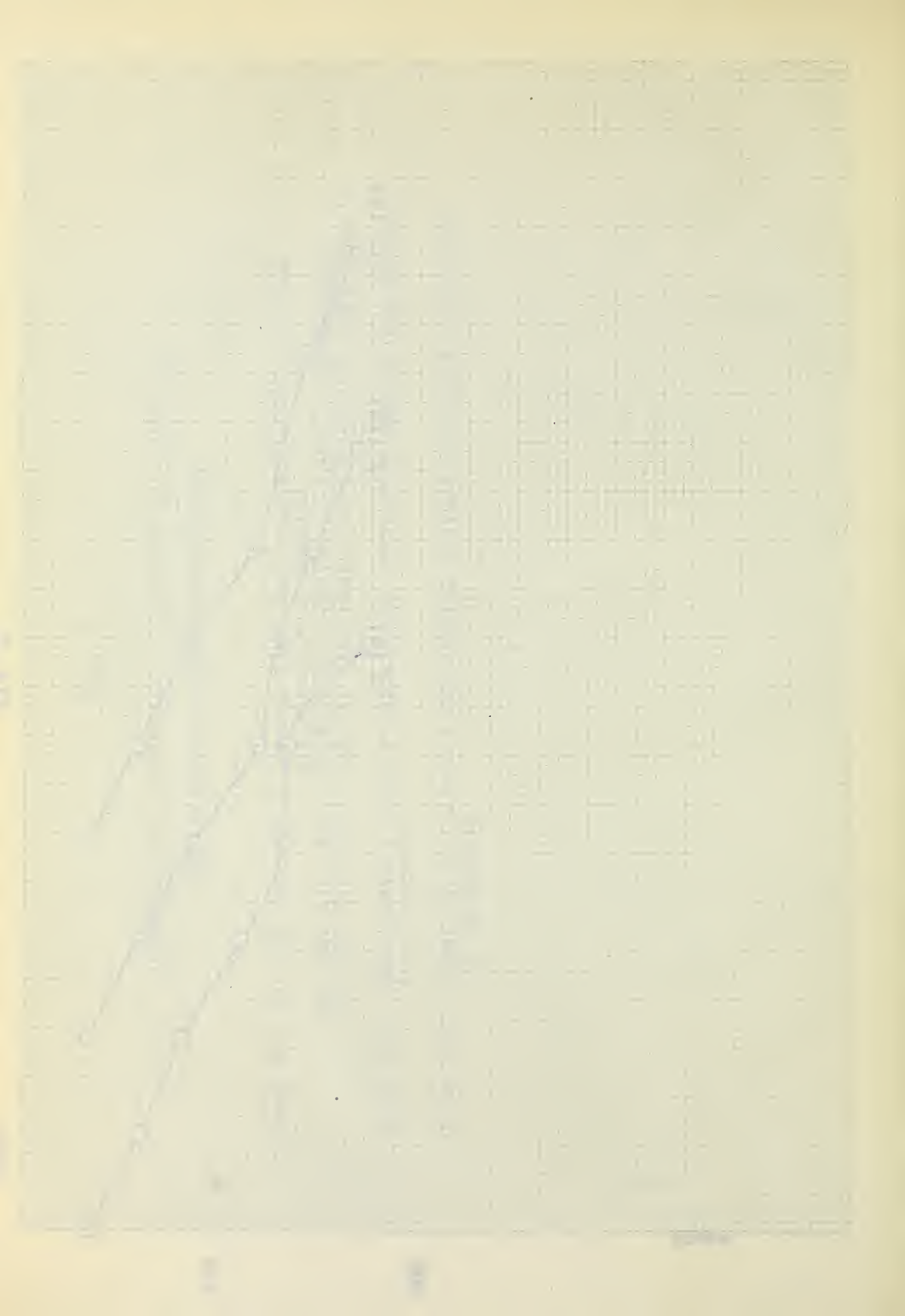


Fig. 8

SHOWING THE GROWTH RESPONSE WITH ALCOHOL EXTRACTS OF MARMITE AND INDICATING QUANTITATIVE EXTRACTION OF VITAMIN B_2 BY THIS PROCEDURE

Group I B - Control Diet

Group IV - Control Diet heated at 105°-120°C.

Group XII - Control Diet heated at 105°-120°C. + 6% Marmite.

Group XIX - Control Diet heated at 105°-120°C. + 60% alcohol extract of Marmite equivalent to 3% Marmite. After three weeks increased to an amount equivalent to 8% Marmite.

Group XX - Control diet heated at 105°-120°C. + 60% Marmite. After three weeks increased to an amount equivalent to 8% Marmite.

Group XXI - Same as group XX, but using an 80% alcohol extract of Marmite equivalent to 8% Marmite.

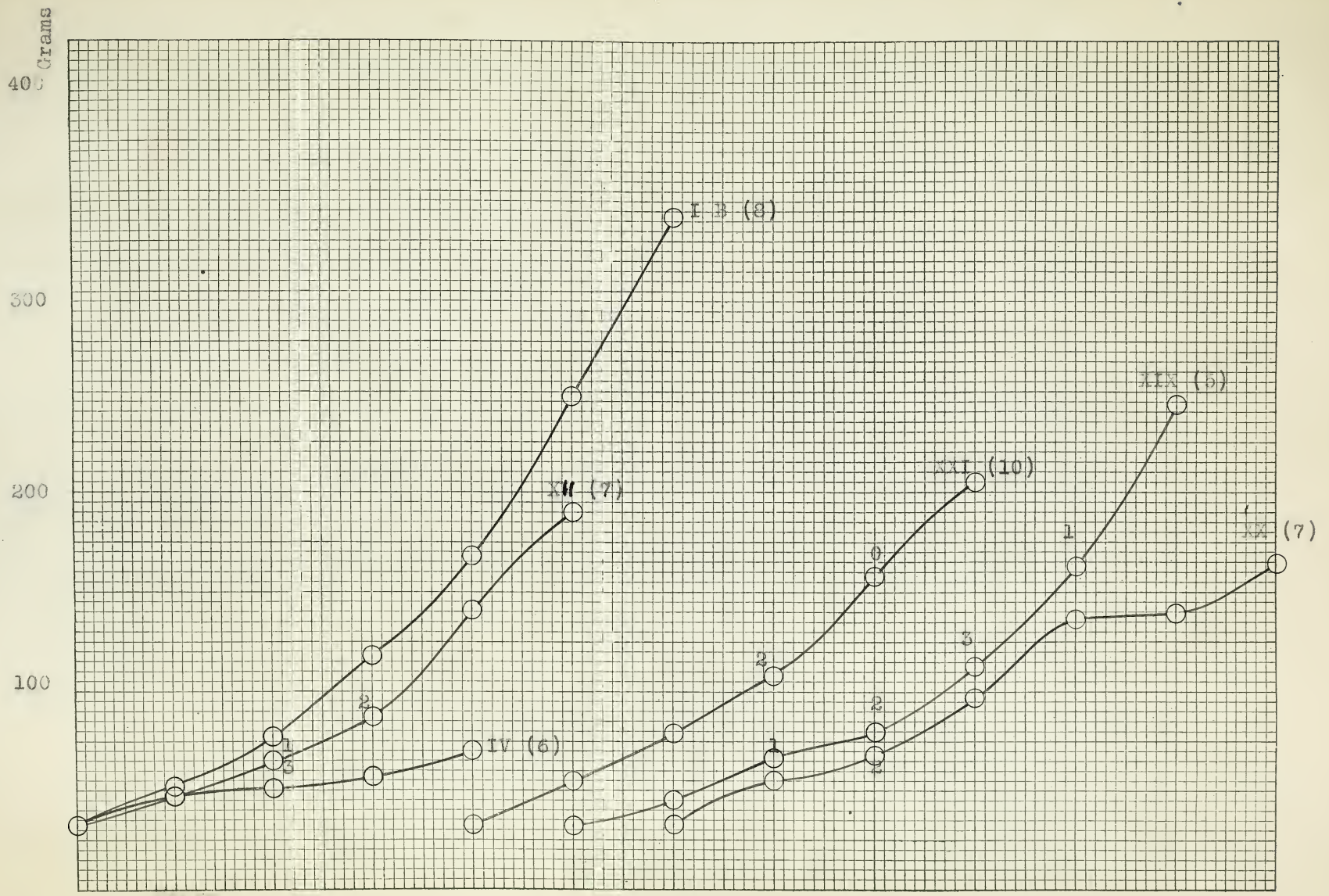


Fig. 8

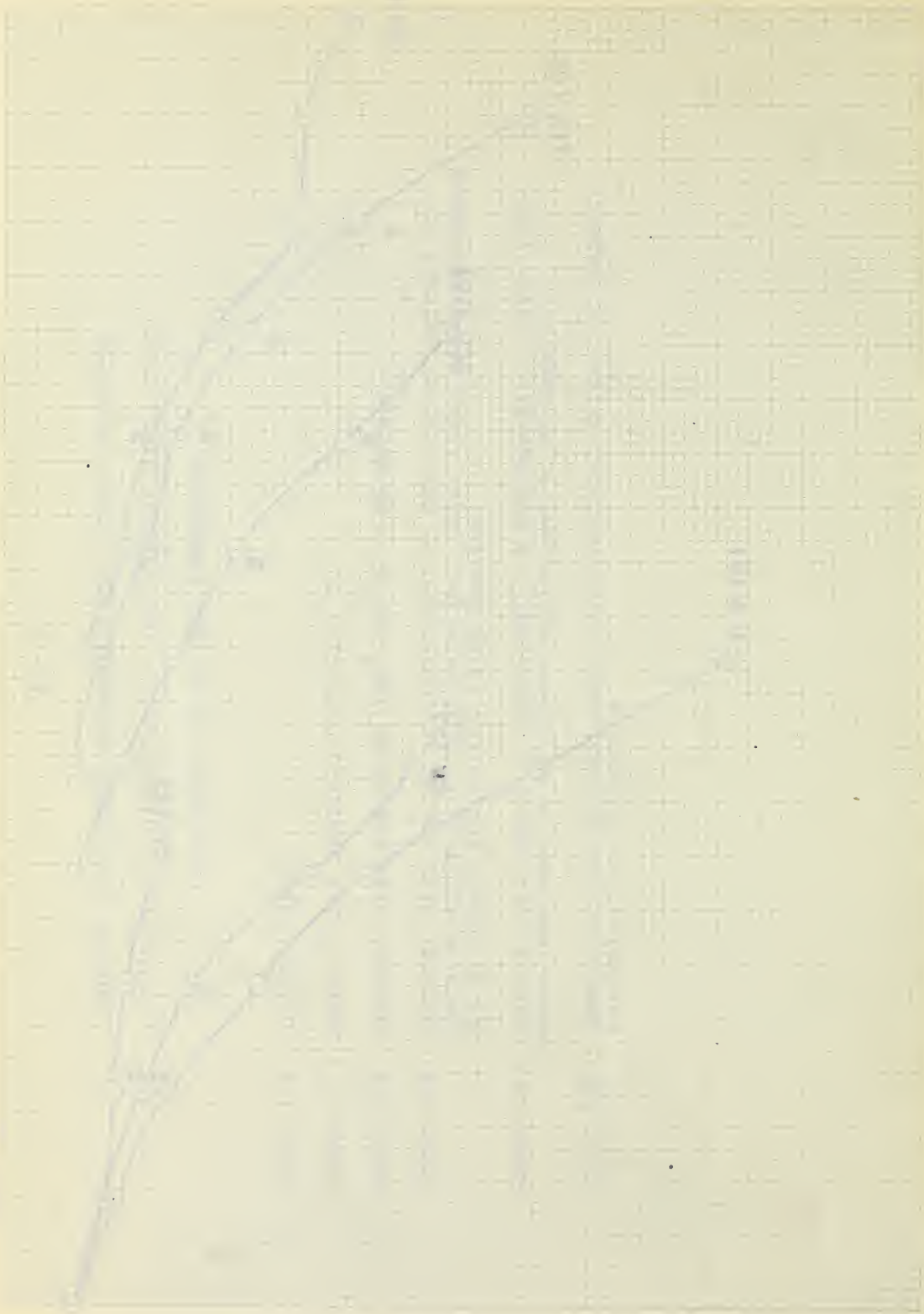


Fig. 9

SHOWING THE RETARDED GROWTH OBTAINED WITH ELUATES PREPARED FROM FULLERS' EARTH ADSORBATES OF ALCOHOL EXTRACTS OF MARMITE.

Group IV - Control diet heated at 105°-120°C.

Group XIX - Control diet heated at 105°-120°C. + 60% alcohol extract of Marmite equivalent to 3% Marmite. After three weeks changed to a supplement equivalent to 8% Marmite.

Group XXIV - Control diet heated at 105°-120°C. + eluate from a 60% alcohol extract of Marmite equivalent to 6% Marmite. After 2nd week eluate increased to equivalent of 12% Marmite.

Group XXV - Control diet heated at 105°-120°C. + eluate of 80% alcohol extract of Marmite in amounts increased as follows:
1st and 2nd week - equivalent to 10% Marmite
3rd week - equivalent to 20% Marmite.
4th week - equivalent to 25% Marmite

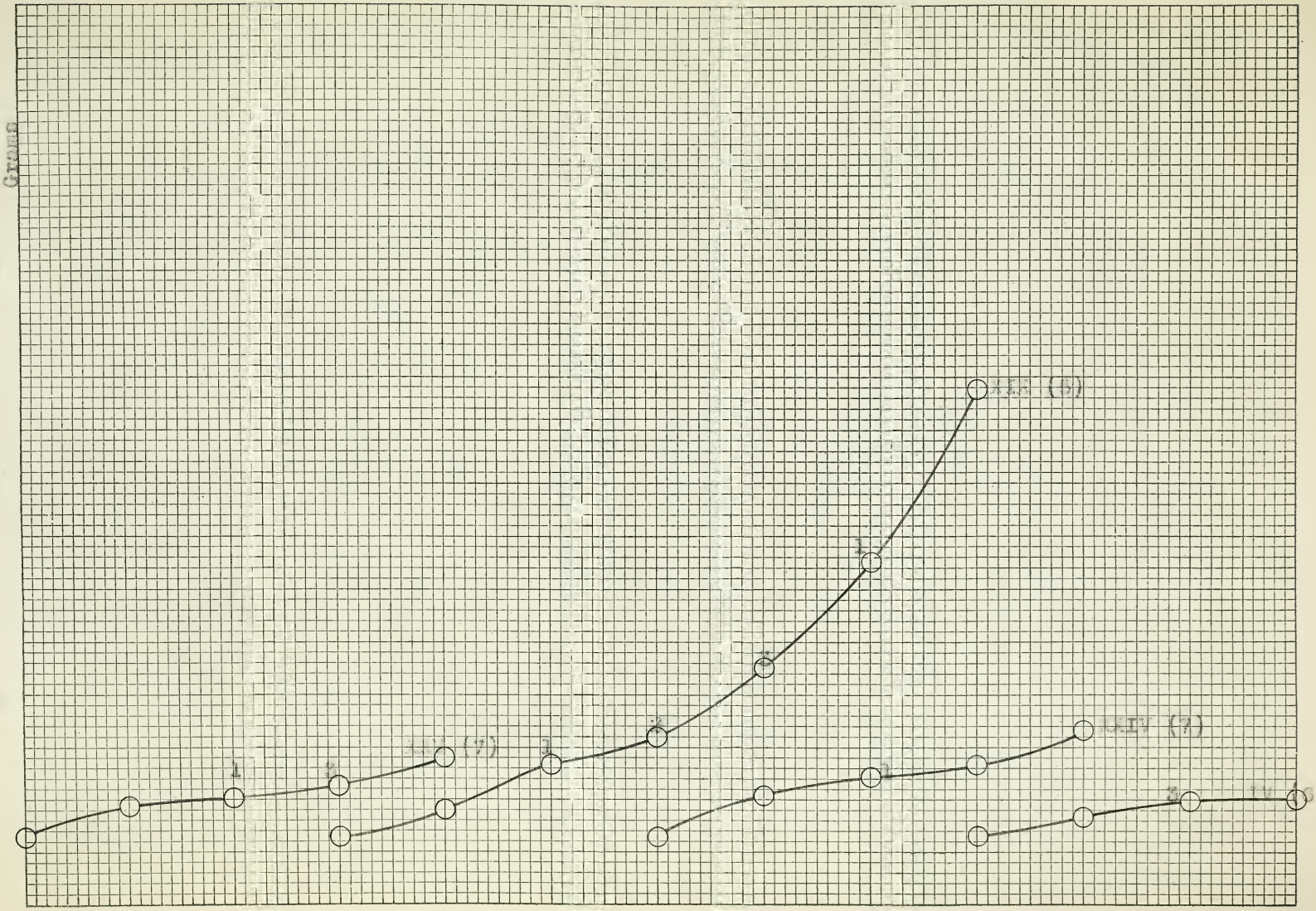


Fig. 9

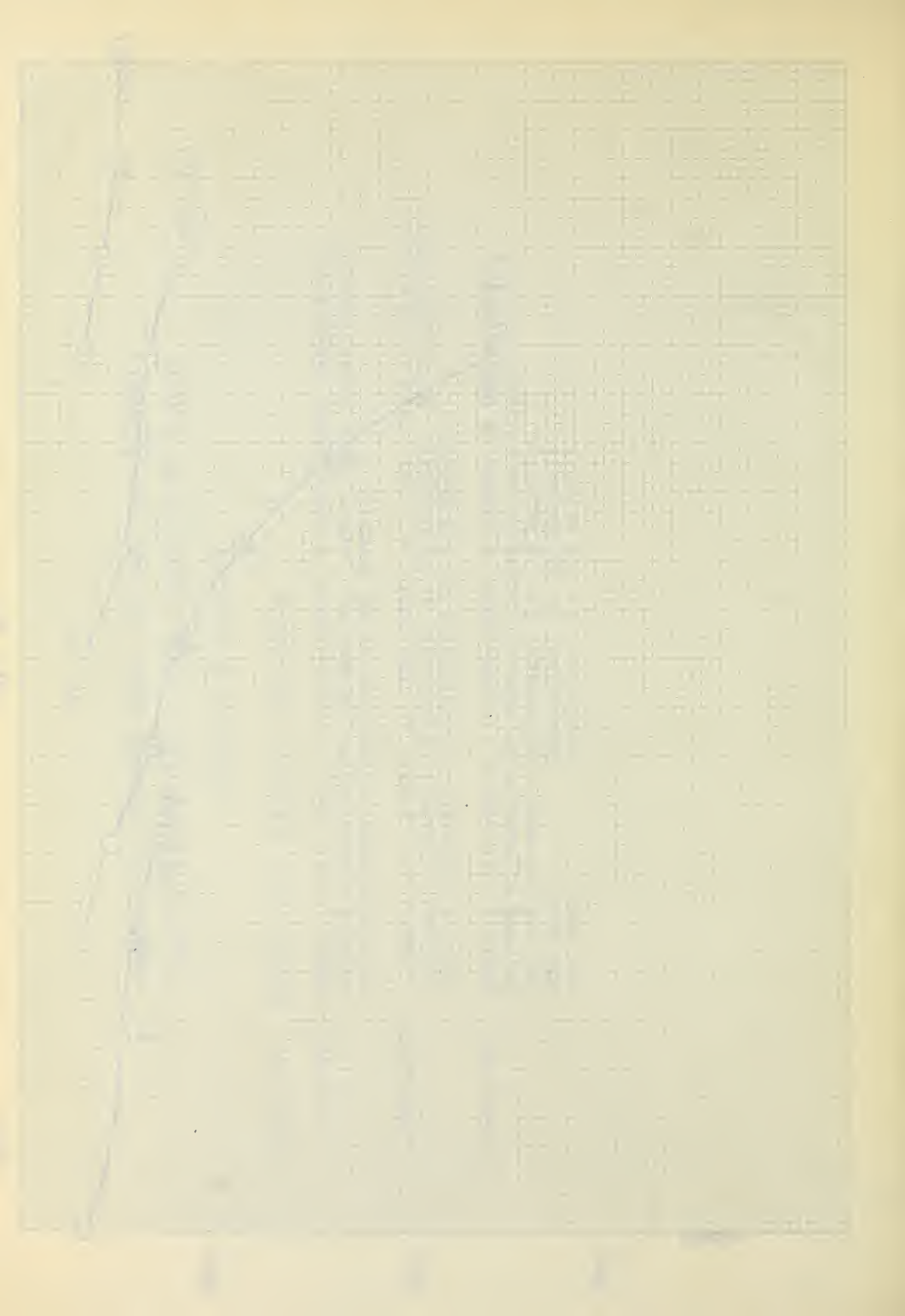


Fig. 10

ILLUSTRATING THE FACT THAT ONLY A PORTION OF THE GROWTH PROMOTING ACTIVITY OF ALCOHOL EXTRACTS OF MARMITE IS CONTAINED IN THE FULLERS' EARTH ADSORBATE.

Group IV - Control diet heated at 105°-120°C.

GGroup XXII - Control diet heated at 105°-120°C. + 80% alcohol extract of Marmite equivalent to 8% Marmite. After two weeks the amount of the alcohol extract increased to the equivalent of 10% Marmite.

Group XXV - Control diet heated at 105°-120°C. + eluate from an adsorbate of an 80% alcohol extract of Marmite.
1st and 2nd week - eluate equivalent to 10% Marmite
3rd week - eluate equivalent to 20% Marmite
4th week - eluate equivalent to 25% Marmite

Group V - Control diet heated at 96°-110°C.

Group XXIII - Control diet heated at 96°-110°C + 80% alcohol extract equivalent to 8% Marmite

Group XXVI - Control diet heated at 96°-110°C. + fullers' earth eluate of an 80% alcohol extract of Marmite equivalent to 12% Marmite.

Group XXVII - Same heated control + fullers' earth adsorption filtrate of an 80% alcohol extract of Marmite equivalent to 12% Marmite.

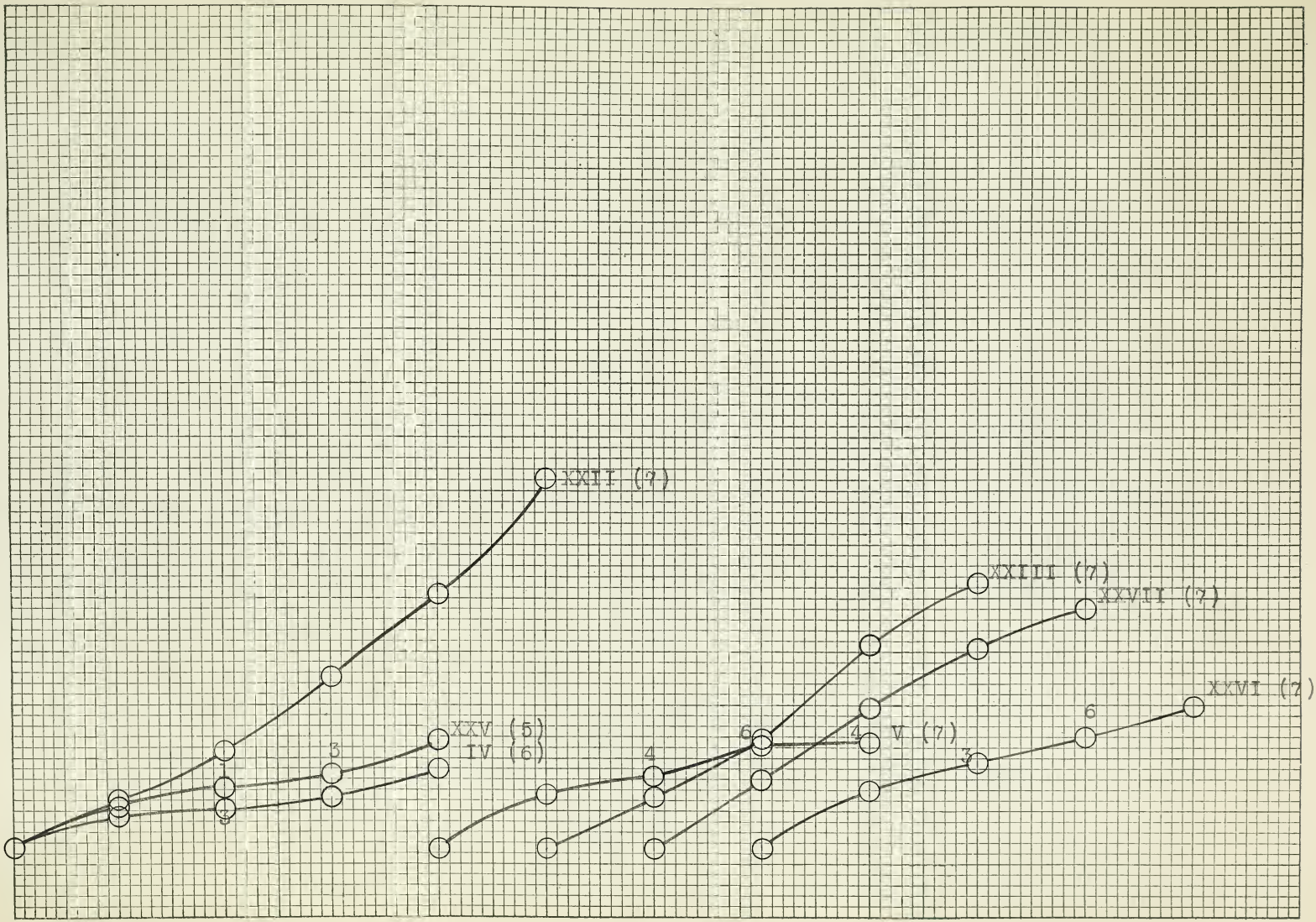

Grams
300
200
100
Weeks
XXII (7)
XXIII (7)
XXVII (7)
XXVI (7)
XXV (5)
IV (6)
V (7)
3
4
6
5

Fig. 10

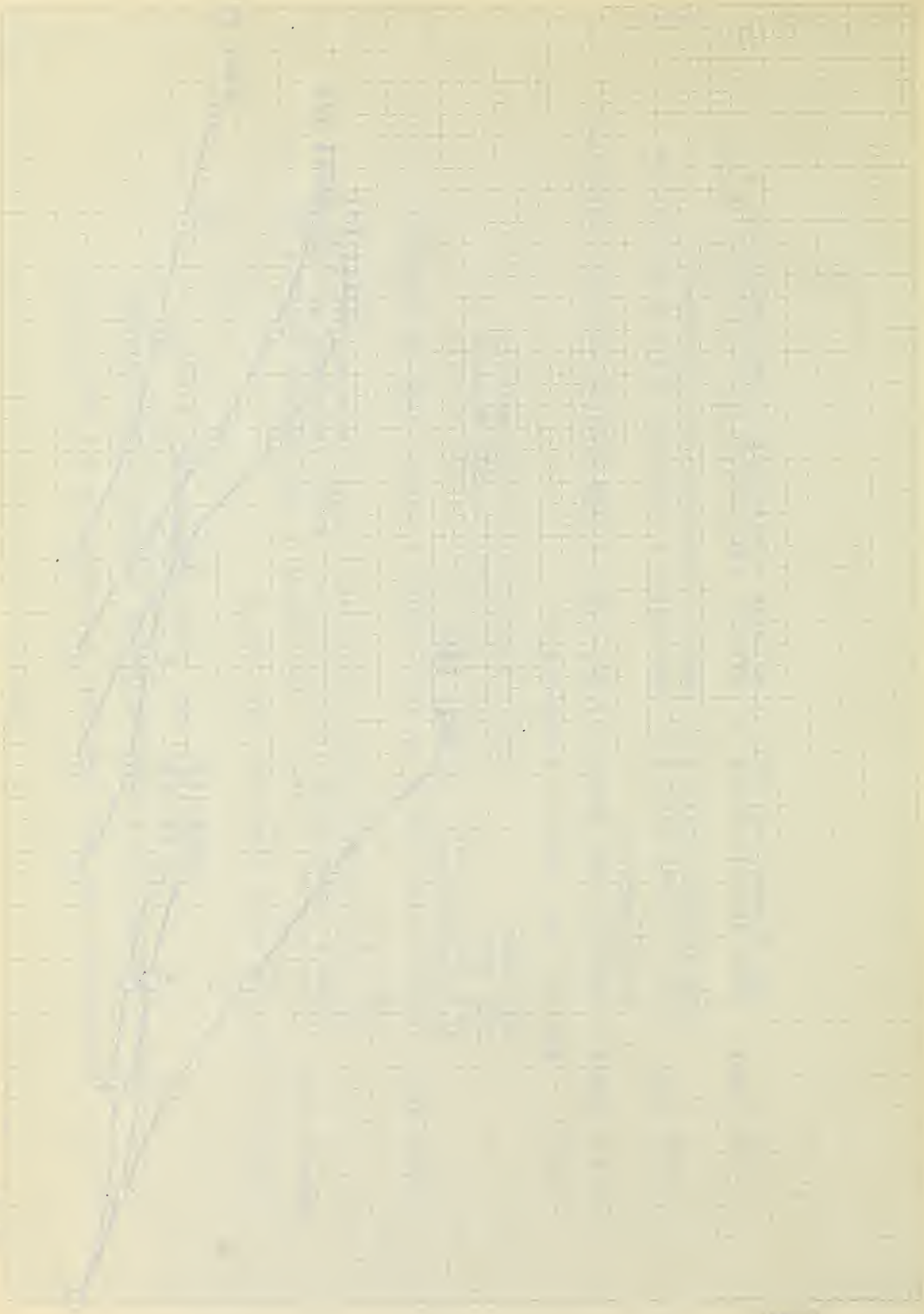


Fig. 11

COMPARING THE GROWTH RESPONSE OBTAINED WITH A FULLERS' EARTH ELUATE WITH THAT OF A LLOYD'S REAGENT ELUATE.

Group V Control diet heated at 96°-110°C.

Group XXVI Control diet heated at 96°-110°C. + fullers' earth eluate of an 80% alcohol extract of Marmite equivalent to 12% Marmite.

Group XXVIII Control diet heated at 96°-110°C. + Lloyd's reagent eluate of an 80% alcohol extract of Marmite equivalent to 12% Marmite.

Group XXIX Control diet heated at 96°-110°C. + Lloyd's reagent adsorption of an 80% alcohol extract of Marmite equivalent to 12% Marmite.

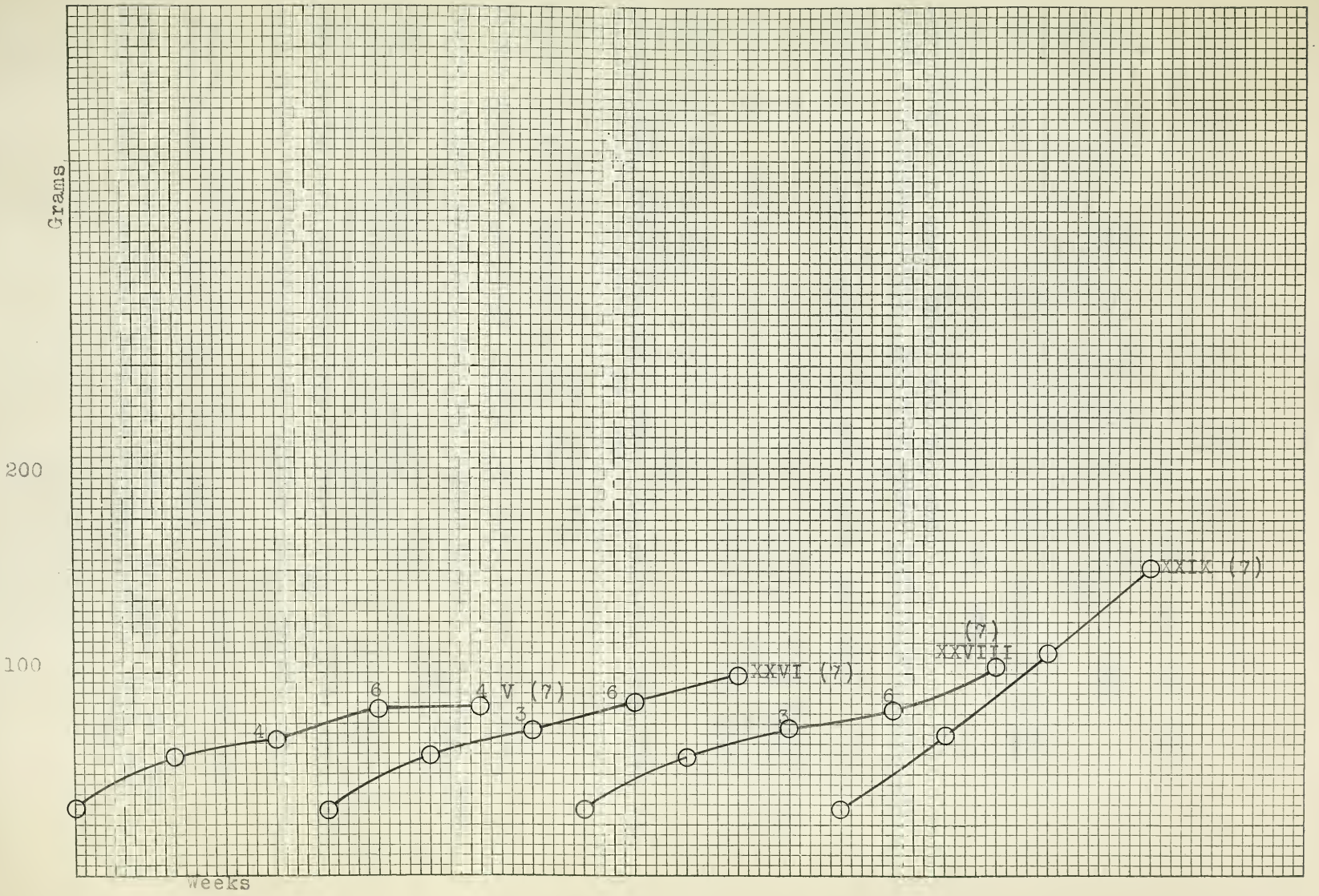
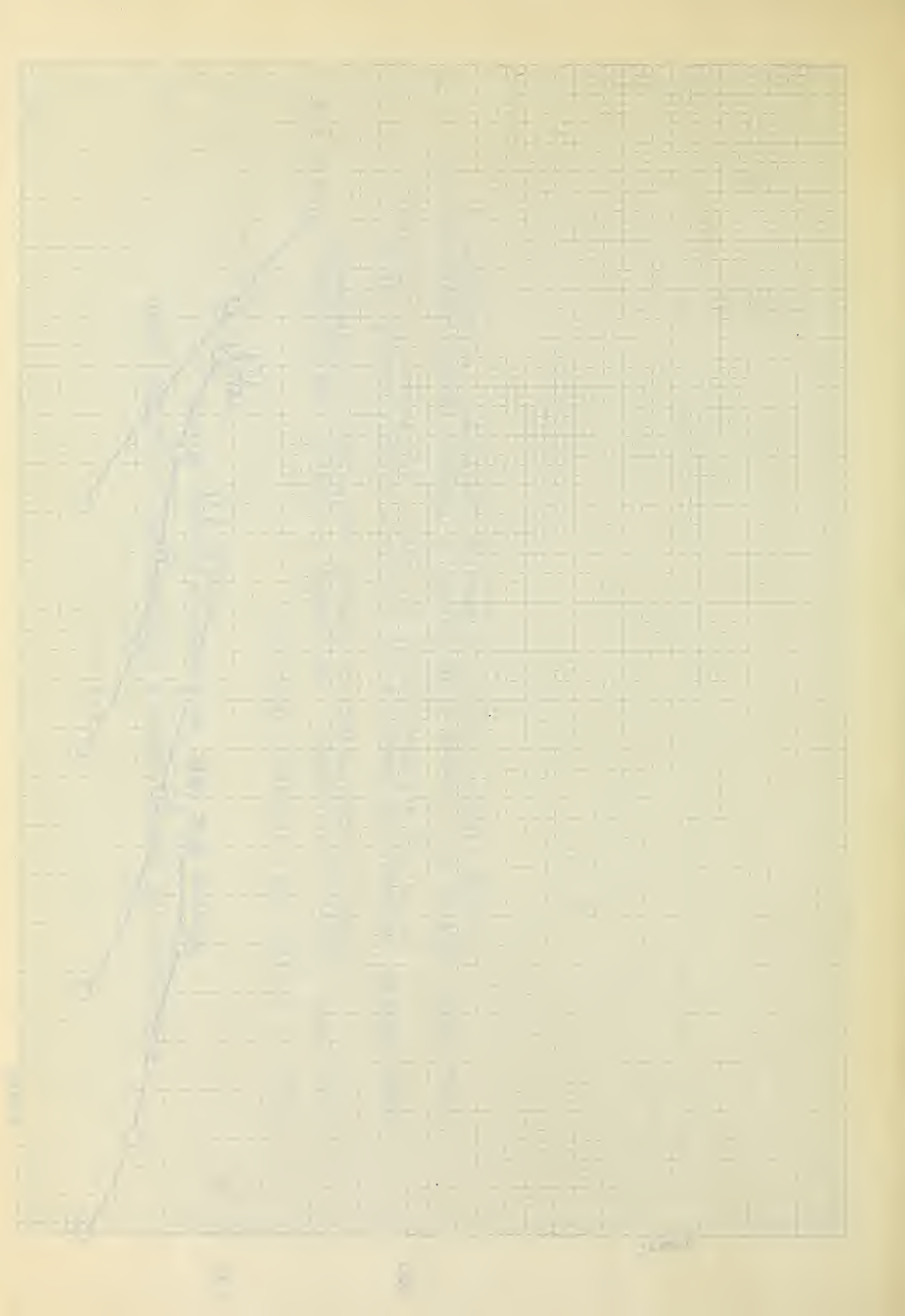


Fig. 11.



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